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Intergrated Analysis of the Multifunctional Protein Aft1p

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Integrated Analysis of the Multifunctional Protein Aft1p

by

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ABSTRACT

The *Saccharomyces cerevisiae* transcription factor Aft1p has an established role under iron-deplete conditions in the induction of genes involved in iron acquisition and utilization. Recent studies have indicated that Aft1p is required for faithful chromosome transmission and *aft1*Δ mutant cells have defects in cell cycle progression. This suggests that Aft1p has additional, but undefined, cellular roles under iron-replete conditions. To explore the role of Aft1p, two genome-wide screens were utilized to identify nonessential deletion mutants that are unable to tolerate defects in Aft1p function, and an Aft1p physical protein-protein interaction map was constructed using affinity purification and mass spectrometry. Our integrative analysis has produced an extended network of genetic and physical interactions, which connect Aft1p to a wide range of cellular functions including the *RIM101* pH response pathway, DNA replication, and DNA repair. Our results establish that Aft1p is multifunctional with broad cellular roles.

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List of Abbreviations:

BPS	Bathophenantroline-disulphonate
cdc	Cell Division Cycle
CEN	Centromere
ChIP	Chromatin Immunoprecipitation
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
GO	Gene Ontology
HU	Hydroxyurea
IRE	Iron-Responsive Element
MMS	Methyl Methanesulfonate
mRNA	Messenger Ribonucleic Acid
NLS	Nuclear Localization Signal
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
SDL	Synthetic Dosage Lethal
SDS	Synthetic Dosage Sickness OR Sodium-Dodecyl Sulfate
SGA	Synthetic Genetic Array
SL	Synthetic Lethal
SS	Synthetic Sickness
TAE	Tris-acetate-EDTA
TAP	Tandem Affinity Purification
YPD	Yeast Peptone Dextrose

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Chapter 1: INTRODUCTION

The single cellular yeast, *Saccharomyces cerevisiae*, has a limited number of proteins, yet it is able to adapt to a multitude of environmental challenges and stresses in order to survive. This suggests that although the yeast genome is finite, many of its proteins are multifunctional in nature. The transcription factor Aft1p is one such multifunctional protein. Aft1p (also known as Rcs1p) is the transcriptional regulator of a sophisticated network that tightly governs iron homeostasis in yeast (*1, 2*). Aft1p is activated in iron-deficient cells to induce the expression of 24 genes, referred to as the "iron regulon" (*3-6*), which increase iron uptake and remodel cellular metabolism to survive low iron conditions (*2*). In addition, recent systematic genetic and phenotype screens have indicated that the cellular role for Aft1p is not restricted to transcriptional responses under limited iron conditions. One study found that Aft1p is required for faithful chromosome transmission fidelity under normal iron conditions (*7*). Another study demonstrated that *aft1Δ* mutants are significantly larger than wild type cells (*8*) suggesting a potential role for Aft1p in cell cycle regulation. These studies suggest that the biological roles of Aft1p are not limited to iron-depleted conditions but that Aft1p may be central to numerous cellular functions under normal iron conditions.

In this study I have utilized and integrated systematic genomic and proteomic technologies to identify and characterize potential iron-independent cellular roles of Aft1p. Two unbiased, genome-wide screens employing Synthetic Genetic Array (SGA) methodology combined with affinity purification and mass spectrometry identification of protein interactions reveal that this multifunctional protein has numerous cellular roles under normal iron conditions in the Rim101 pH response pathway, cell cycle regulation, and DNA

damage repair. Overall, this study demonstrates that a better understanding of multifunctional proteins is critical to elucidating the holistic view of the cell and complex cellular diseases.

Literature Review

I. Aft1p, an iron-responsive transcription factor in yeast

i. Iron in the cell

Iron is an essential mineral required by almost all organisms. Its ability to easily gain or lose electrons makes it an important co-factor in diverse enzymatic reactions (*9*), including cellular respiration and iron-sulfur biosynthesis (*1*). However, given that perturbations in cellular iron levels can result in toxicity, contributing to numerous disease phenotypes, tight regulation of iron uptake and utilization is required within the cell (*2*). For example, iron-overload can result in free-radical production, a type of oxidative stress that is present in many forms of cancer (*10*), whereas iron-deficiency can result in numerous types of anemia, such as the blood transfusion-dependent disorder thalassemia (*11*). Iron chelators are currently being used as therapeutic agents for the treatment of both of these diseases (*11*, *12*) demonstrating the importance of understanding the molecular mechanisms governing iron regulation. Systematic identification of the pathways and proteins that are sensitive to cellular iron perturbations will provide a better understanding of the relationship between cellular iron levels and iron-related disease progression.

ii. Iron response in yeast

Like all organisms, the yeast *Saccharomyces cerevisiae* maintains tight regulation of cellular iron uptake and utilization in order to prevent toxicity caused by iron overload (*2*). Free iron is taken into the cell by two distinct iron-transport systems, the high- and low-affinity iron transport systems, depending on the bioavailability of the metal (*1*). In the environment, iron is naturally found in the ferric form (Fe^{3+}) and the cell-surface reductases,

Fre1p and Fre2p, reduce ferric iron to provide ferrous iron (Fe^{2+}) for both transport systems. When cellular iron levels are high, the low-affinity transport system (K_m= 30 μM), composed of Fet4p, a non-ATP-dependent transmembrane transporter, is used to import iron into the cell. Under low iron conditions, the high-affinity transport system (K_m=0.15 μM), composed of a complex between the integral membrane ferroxidase Fet3p and the iron permease Ftr1p, is used to import iron across the membrane (1, 15, 13).

iii. Aft1p, a regulator of the high affinity iron transport system

S. cerevisiae responds to low iron conditions through transcriptional remodeling governed primarily by the iron-responsive transcription factor Aft1p (for "activator of ferrous transport") (*1*). Upon iron-depletion, Aft1p binds to iron-responsive elements (IREs), with the consensus sequence 5'-PyPuCACCCPu-3' (4, 14), in the promoters of 24 genes referred to as the "iron regulon" (see Table 1) (*3-6*). The resultant transcriptional activation of these genes causes an increase in iron uptake and remodels cell metabolism to survive low iron conditions (*2*).

Genes of the iron regulon form an ordered and sophisticated regulatory network. The cell-surface ferric reductases (*FRE1, FRE2,* and *FRE3*) reduce ferric iron (Fe^{3+}) found in the environment, to provide ferrous iron (Fe^{2+}) as a substrate for the high-affinity transport system. This system is composed of a complex between the integral membrane ferroxidase Fet3p and the iron permease Ftr1p. Iron can also be acquired from siderophores, which are low-molecular-weight organic molecules that chelate iron, that are produced by microorganisms in the surrounding environment. The transporters (*ARN1-4*) mediate siderophore uptake by cycling between the cell surface and an endosomal compartment and

Table 1. Genes of the iron regulon

Function	Gene name	Regulated by Aft1p	Regulated by Aft2p
Transporters	*FET5*	yes	yes
	FTR1	yes	yes
	FTH1	yes	no
	SMF3	yes	yes
	MRS4	yes	yes
	CCC2	yes	no
Cu chaperone	*ATX1*	yes	yes
Ferroxidase	*FET3*	yes	yes
	FET5	yes	yes
Metalloreductases	*FRE1*	yes	yes
	FRE2	yes	no
	FRE3	yes	no
	FRE4	yes	no
	FRE5	yes	no
	FRE6	yes	no
Cell wall proteins	*FIT1*	yes	yes
	FIT2	yes	yes
	FIT3	yes	yes
Siderophore transport	*ARN1*	yes	no
	ARN2	yes	no
	ARN3	yes	no
	ARN4	yes	no
Fe-S biosynthesis	*ISU1*	yes	yes
	ISU2	yes	no
Other	*TIS11*	yes	yes

Adapted from (1)

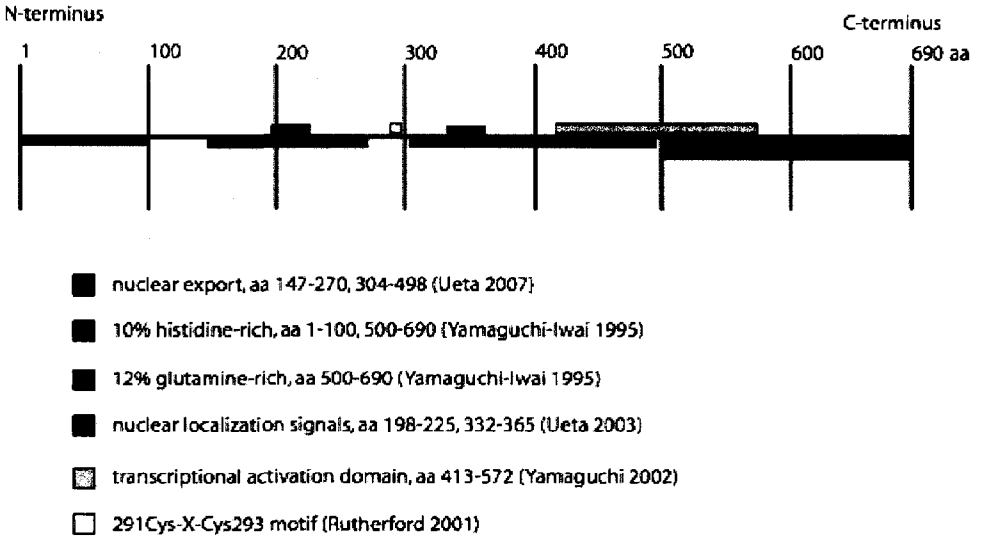
the mannoproteins (*FIT1-3*) sequester this iron chelate within the cell wall. Other genes involved in the high affinity transport system include the vacuolar iron transporter (*FET5/FTH1*), genes involved in copper delivery to the Fet3p oxidase (*ATX1* and *CCC2*), and *TIS11*, a gene that specifically binds to and degrades mRNAs that encode proteins involved in iron-dependent activities (*1, 15*).

iv. Aft1p structure and localization

AFT1 encodes a protein of 690 amino acids (depicted in Fig.1) (*16*) that is constitutively expressed, and under normal iron conditions, is routinely shuttled between the nucleus and the cytoplasm (*17*). Nuclear import of Aft1p is independent of cellular iron levels and is mediated by two nuclear localization signals (NLS) at amino acids 198-225 and 332-365 as well as a direct interaction with the nuclear import factor Pse1p (*17*). Export of Aft1p from the nucleus is promoted in the presence of iron and is mediated by two regions, amino acids 147-270 and 304-498 (*18*). The N-terminal region of Aft1p is believed to act as a DNA binding domain, due to its highly basic nature (*19*) that is characteristic of DNA recognition sequences in other proteins (*20*). The C-terminal region, between amino acids 413-572 (*21*) is believed to act as the transcriptional activation domain, due to its glutamine-rich nature (*19*), a feature that is found in a number of transcriptional activation domains (*22*).

The exact mechanism by which Aft1p senses cellular iron levels is not understood. It has been hypothesized that Aft1p may bind directly to iron and the loss of iron binding could initiate Aft1p response (*1*), however Aft1p has not been shown to bind iron or an iron sulfur cluster to date (*23*). Four cysteine residues (Cys143, 215, 291, 293) reside in the middle of

Figure 1. Structural features of *AFT1*.
Schematic of the known domains of Aft1p. Aft1p is 690 amino acids in length with two histidine-rich regions (red) residing at the N- and C-termini. There are two nuclear localization signals required for nuclear import (blue), and two regions required for nuclear export (green). The transcriptional activation domain (orange) and the glutamine-rich region (purple) are where DNA binding occurs. The Cys-X-Cys motif in the middle of the molecule (yellow) is hypothesized to bind iron. References are located at the right hand side of the legend.


N-terminus
C-terminus
1
100
200
300
400
500
600
690 aa
nuclear export, aa 147-270, 304-498 (Ueta 2007)
10% histidine-rich, aa 1-100, 500-690 (Yamaguchi-Iwai 1995)
12% glutamine-rich, aa 500-690 (Yamaguchi-Iwai 1995)
nuclear localization signals, aa 198-225, 332-365 (Ueta 2003)
transcriptional activation domain, aa 413-572 (Yamaguchi 2002)
291Cys-X-Cys293 motif (Rutherford 2001)

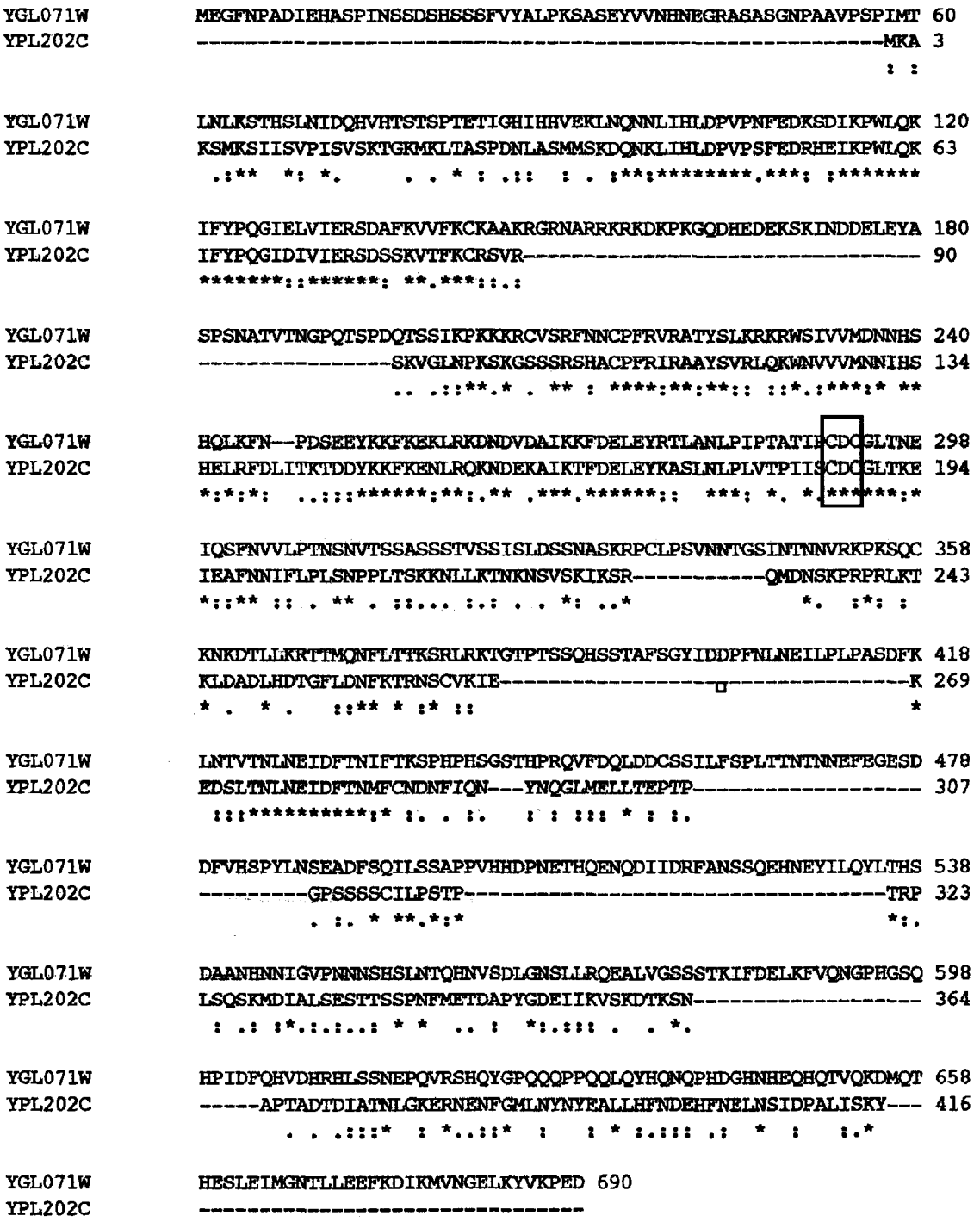
the molecule (*24*), with the ^{291}Cys-X-Cys^{293} motif (depicted in Fig. 1) hypothesized to be involved in the binding of iron (*5*). This hypothesis is based on the observation that a cysteine-to-phenylalanine mutation at residue 291 (*AFT1-1^{up}*) results in constitutive localization of Aft1p to the nucleus and constitutive expression of genes of the iron regulon, irrespective of cellular iron levels (*5, 19*).

v. Aft2p, a paralog of Aft1p

In the absence of Aft1p, its paralog Aft2p, can also regulate transcription of genes involved in iron acquisition and utilization (*3, 5, 24*). Aft1p and Aft2p share significant regions of identity, with 39% sequence similarity (*24*) (Fig. 2), and are thought to have arisen from a genome duplication event (*25*). Some of the similarities between these two proteins include their highly conserved N-terminal region (*19*), the four conserved cysteine residues in the middle of each molecule (*24*), and their shared ability to bind to and transcriptionally activate genes containing iron-responsive elements in their promoters (*4, 14*).

Although these two proteins have overlapping functions, their roles in the transcriptional regulation of the iron regulon are non-redundant (*24*). For example, microarray experiments with an *aft1Δaft2Δ* double mutant constitutively expressing either Aft1p (*Aft1-1^{up}*) or Aft2p (*Aft2-1^{up}*) in the nucleus demonstrate that Aft1p has a more prominent role than Aft2p in the transcriptional activation of the iron regulon, with each factor generating a distinct transcriptional profile (*4*). It has been hypothesized that these differences may be attributed to these two proteins having different *cis* regulatory elements (*3*). Additionally *aft1Δ* and *aft2Δ* mutants display distinct phenotypes, where an *aft1Δ* null mutant exhibits low ferrous iron uptake and poor growth under low-iron conditions, and an

Figure 2. *AFT1* and *AFT2* sequence alignment.
Sequence comparison of Aft1p (*YGL071W*) and Aft2p (*YPL202C*). The amino acid sequences of Aft1p and Aft2p of *S. cerevisiae* were aligned using the CLUSTALW program. The red box outlines the conserved Cys-X-Cys motif.


YGL071W MEGFNPADIEHASPINSSDSHSSSFVYALPKSASEYVVNHNEGRASASGNPAAVPSPIMT 60
YPL202C ----------MKA 3
YGL071W LNLKSTHSLNIDQHVHTSTSPTETIGHIHHVEKLNQNNLIHLDPVPNFEDKSDIKPWLQK 120
YPL202C KSMKSIISVPISVSKTGKMKLTASPDNLASMMSKDQNKLIHLDPVPSFEDRHEIKPWLQK 63
YGL071W IFYPQGIELVIERSDAFKVVFKCKAAKRGRNARRKRKDKPKGQDHEDEKSKINDDELEYA 180
YPL202C IFYPQGIDIVIERSDSSKVTFKCRSVR---------- 90
YGL071W SPSNATVTNGPQTSPDQTSSIKPKKKRCVSRFNNCPFRVRATYSLKRKRWSIVVMDNNHS 240
YPL202C ----------SKVGLNPKSKGSSSRSHACPFRIRAAYSVRLQKWNVVVMNNIHS 134
YGL071W HQLKFN--PDSEEYKKFKEKLRKDNDVDAIKKFDELEYRTLANLPIPTATIRCDCGLTNE 298
YPL202C HELRFDLITKTDDYKKFKENLRQKNDEKAIKTFDELEYKASLNLPLVTPIISCDCGLTKE 194
YGL071W IQSFNVVLPTNSNVTSSASSSTVSSISLDSSNASKRPCLPSVNNTGSINTNNVRKPKSQC 358
YPL202C IEAFNNIFLPLSNPPLTSKKNLLKTNKNSVSKIKSR----------QMDNSKPRPRLKT 243
YGL071W KNKDTLLKRTTMQNFLTTKSRLRKTGTPTSSQHSSTAFSGYIDDPFNLNEILPLPASDFK 418
YPL202C KLDADLHDTGFLDNFKTRNSCVKIE----------K 269
YGL071W LNTVTNLNEIDFTNIFTKSPHPHSGSTHPRQVFDQLDDCSSILFSPLTTNTNNEFEGESD 478
YPL202C EDSLTNLNEIDFTNMFCNDNFIQN---YNQGLMELLTEPTP---------- 307
YGL071W DFVHSPYLNSEADFSQILSSAPPVHHDPNETHQENQDIIDRFANSSQEHNEYILQYLTHS 538
YPL202C ----------GPSSSSCILPSTP----------TRP 323
YGL071W DAANHNNIGVPNNNSHSLNTQHNVSDLGNSLLRQEALVGSSSTKIFDELKFVQNGPHGSQ 598
YPL202C LSQSKMDIALSESTTSSPNFMETDAPYGDEIIKVSKDTKSN---------- 364
YGL071W HPIDFQHVDHRHLSSNEPQVRSHQYGPQQQPPQQLQYHQNQPHDGHNHEQHQTVQKDMQT 658
YPL202C -----APTADTDIATNLGKERNENFGMLNYNYEALLHFNDEHFNELNSIDPALISKY--- 416
YGL071W HESLEIMGNTLLEEFKDIKMVNGELKYVKPED 690
YPL202C ----------

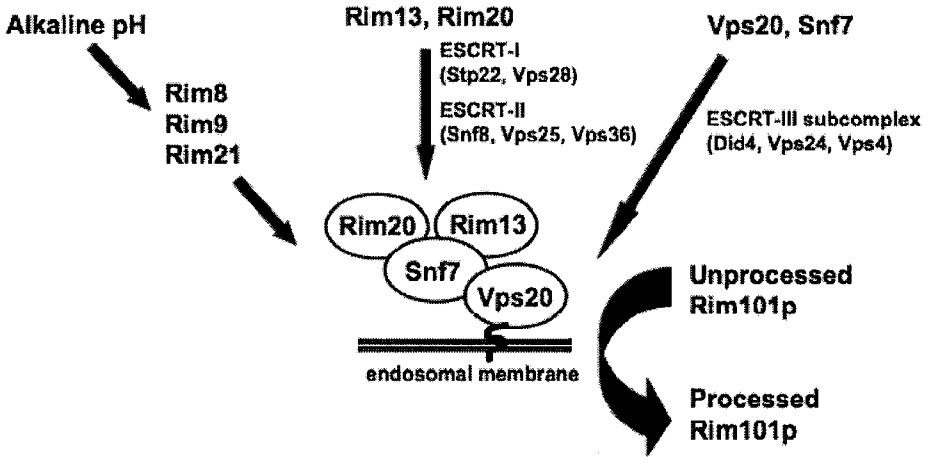
aft2Δ null mutant shows no phenotype under these conditions (*26*). This suggests that Aft1p is the primary mediator of iron regulation within the cell. However, consistent with the functional similarity of these two proteins, an *aft1Δaft2Δ* double mutant is more sensitive to low-iron growth than a single *aft1Δ* null mutant alone (*5, 24*).

vi. Cross-talk between iron and RIM101 pH-responsive pathways

There has been some indication of cross-talk between the cellular pathways governing iron regulation and pH response in yeast. Several microarray studies have shown that upon alkaline pH treatment, expression levels of genes involved in iron acquisition and utilization are increased (*27*) (*28, 29*), however it is unclear whether this transcriptional response is dependent on Aft1p. Additionally, it has been shown that *aft1Δ* mutants show hypersensitivity to alkaline pH conditions (*30*) further suggesting that there is cross-talk between these two cellular pathways by a yet unidentified mechanism.

The pH response or Rim101 pathway in yeast is mainly governed by the transcription factor, Rim101p (see schematic of pathway in Fig. 3 and a detailed list of the genes and their functions in Table 4) (*31*). Rim101p is a transcriptional repressor that negatively regulates expression of several target genes involved in the response to alkaline pH. Upon alkaline treatment the transmembrane proteins Rim8p, Rim9p, and Rim21p, through an unresolved mechanism, recruit the "class E" vacuolar protein sorting ESCRT-III components, proteins Snf7p and Vps20p, to the endosomal membrane. Accumulation of these two proteins at the membrane, with the aid of the ESCRT-I and –II subcomplexes, in turn recruits the protease scaffold Rim20p and the calpain-like protease Rim13p. This results in the formation of a Rim20p-(Vps20p-Snf7p)-Rim13p complex, leading to catalytic cleavage of the C-terminal

Figure 3. Schematic illustration of the Rim101 pathway.
Model for activation of Rim101 proteolytic processing by components of the Rim101 pathway upon exposure to alkaline pH. Upon alkaline conditions the transmembrane proteins Rim8, 9, and 21, through an unresolved mechanism, recruit the "class E" vacuolar protein sorting ESCRT-III components, proteins Snf7p and Vps20p, to the endosomal membrane. Accumulation of these two proteins at the membrane, with the aid of the ESCRT-I and –II subcomplexes, in turn recruits the protease scaffold Rim20p and the calpain-like protease Rim13p. This results in the formation of a Rim20p-(Vps20p-Snf7p)-Rim13p complex, leading to catalytic cleavage of the C-terminal inhibitory domain of Rim101p, its subsequent release from the promoters of inhibited genes, and the resultant transcription of genes involved in alkaline pH response (adapted from(1)).


Alkaline pH
Rim8
Rim9
Rim21
Rim13, Rim20
ESCRT-I
(Stp22, Vps28)
ESCRT-II
(Snf8, Vps25, Vps36)
Vps20, Snf7
ESCRT-III subcomplex
(Did4, Vps24, Vps4)
Rim20
Rim13
Snf7
Vps20
endosomal membrane
Unprocessed
Rim101p
Processed
Rim101p

inhibitory domain of Rim101p, its subsequent release from promoters, and the resultant transcription of genes involved in alkaline pH response(*31*). This cellular pathway not only governs alkaline pH response in the cell, it also contributes to sporulation, as *RIM101* null mutants are unable to sporulate (*28*), and cell wall assembly, as *rim101Δ* mutants cannot tolerate the deletion of other genes involved in cell wall assembly (*32*). Although the literature suggests that there is cross-talk between iron regulation and the pH response pathway, it is unclear whether Aft1p or iron is required for the additional cellular processes governed by Rim101p, such as sporulation and cell wall assembly.

II. Iron-replete roles for Aft1p

i. Transcription

Though Aft1p has a well-established role in iron-responsive transcription, there has been some indication that Aft1p may have a transcriptional role under iron-replete conditions. For example, a summary of genes activated in microarray experiments using an *aft1Δaft2Δ* double mutant constitutively expressing either Aft1p (*Aft1-1^{up}*) or Aft2p (*Aft2-1^{up}*) in the nucleus demonstrate that only a subset of Aft1p target genes have a known role in iron homeostasis (*4*). More recently, it has been shown that *AFT1* overexpression results in transcriptional modulation of more than 200 genes implicated in a variety of processes, such as vacuolar protein catabolism, the tricarboxylic acid cycle, and biotin uptake and biosynthesis, and that many of these genes are not transcriptionally induced under low-iron conditions (*6*). Genome-wide chromatin immunoprecipitation (ChIP) arrays or ChIP-on-ChIP studies have not successfully mapped Aft1p chromosomal locations (*33*, *34*), therefore it is not known how many of the Aft1p-induced genes are directly regulated by Aft1p

(through binding to IREs in their promoters) or result from downstream transcriptional cascades. These findings demonstrate that the transcriptional role of Aft1p under normal iron conditions requires further investigation.

ii. Cell cycle

Several studies, performed under normal iron conditions, have suggested that Aft1p may play a role in cell cycle regulation. For example, *aft1Δ* mutant cells are significantly larger than wild type cells (*8*), suggesting that these mutant cells have a delay when progressing into "Start" or the G_1/S phase of the cell cycle compared to wild type cells. However it is presently unknown exactly when in the cell cycle or by what mechanism *aft1Δ* mutants undergo this delay. Additionally, overexpression of *AFT1* or *aft1-1*, a mutation that makes Aft1p constitutively localized to the nucleus, results in G1 arrest due to the inhibition of translation of G_1 cyclins by an undetermined mechanism (*26, 35*). It has been shown that Aft1p is also required for cellular resistance to hydroxyurea (HU), an inhibitor of ribonucleotide reductase and DNA replication (*36*). HU treatment results in the Aft1p-dependent induction of the iron regulon and *aft1Δ* mutants display hypersensitivity to HU that can be suppressed by exogenous iron. All of these studies indicate that Aft1p contributes to cell cycle regulation under normal iron conditions by a yet undiscovered mechanism.

iii. Chromosome stability

Recently, a role for Aft1p in chromosome stability has been discovered. Synthetic Genetic Array (SGA) technology, a method that allows the systematic identification of genetic interactions using the yeast gene deletion collection (*37*), identified nonessential

deletion mutants that were unable to tolerate either overexpression or loss of function of kinetochore genes under normal iron conditions (*7*). Most of the identified genes had previously known roles in chromosome segregation. Surprisingly *AFT1* was identified in this series of genome-wide screens, suggesting that this gene has a previously unappreciated role in chromosome stability. Chromosome transmission fidelity assays performed under iron replete conditions determined that *aft1Δ* mutant cells display a 22-fold increase in chromosome loss compared to a wild type control and confirmed that Aft1p does indeed have a central role in chromosome stability (*7*). Furthermore, this study demonstrated that Aft1p co-localizes with kinetochore proteins to centromere (*CEN)* regions (*7*). Interestingly, *AFT2*, the paralog of *AFT1,* or other iron regulon genes were not identified in the genome-wide screens, suggesting that the cellular role of Aft1p in chromosome stability is likely independent of Aft1p's role in iron homeostasis. Presently the molecular mechanism by which Aft1p contributes to genome maintenance is unknown.

iv. DNA damage

A recent genome-wide study assayed the effects of 12 closely related DNA-damaging agents on the yeast haploid gene deletion mutant array. This study found that *aft1Δ* mutants are hypersensitive to inter-strand cross-linking DNA-damaging agents, with the strongest sensitivity to carboplatin, a derivative of cisplatin (*38*). Deletion mutants of other members of the iron regulon or *aft2Δ* did not display similar hypersensitivity to carboplatin in this screen. This suggests that the role of Aft1p in DNA damage response may be fully or partially iron-independent. Alternatively, this hypersensitivity of *aft1Δ* mutants to carboplatin may reflect the dominant role of Aft1p in mediating the full iron-regulon

response. A similar dominant phenotype is seen for *aft1Δ* mutants relative to *aft2Δ* mutants (and other iron-regulon mutants) with respect to iron sensitivity (*24*). Indeed, this may be the case as a recent microarray study demonstrated that 14 genes involved in iron homeostasis, all regulated by Aft1p, have increased mRNA expression levels upon treatment with cisplatin (*39*). Though it is not known if cisplatin and carboplatin display identical cellular transcriptional responses, it is likely that iron levels may contribute to inter-strand cross-linking DNA damage response in yeast, possibly through DNA damage repair genes that require iron for activity. For example, Rad3p, a DNA helicase involved in nucleotide excision repair and transcription in yeast, has an essential iron sulfur domain required for its response to DNA damage within the cell (*40*). Overall, these studies suggest that Aft1p may play a role in DNA damage repair, however the mechanism has yet to be understood.

III. Systems biology

i. A holistic approach to understanding the cell

Systems biology involves studying biological systems on a global level through the application and integration of high-throughput, genome-wide scale methodologies (*41*). These methods enable researchers to study the cell at various levels of complexity such as the genome, the transcriptome, the proteome, and the metabolome (*42*), however the integration of large and disparate data types using bioinformatics tools makes this field of research quite challenging (*41*). Yeast has proven to be a well-defined system for comprehensive studies using systems biology techniques due to a number of favorable characteristics such as: simple methods of cultivation under controlled conditions, well established techniques for genetic manipulation, high similarity between the yeast genome

and higher ordered organisms, and the wealth of resources, databases, and methods for global analysis available to yeast researchers (*42*). In this study, I have employed two systems biology methodologies, functional genomics and protein-protein interaction networks, to investigate the cellular roles of Aft1p under normal iron conditions. It has been well established that genetic interaction networks and physical interaction networks are complementary techniques, and the integration of these data sets can reveal pathway organization and function.

ii. Functional genomics

Genetic interactions are a powerful tool for predicting function. The development of systematic genetic array (SGA) analysis and the yeast deletion mutant array has enabled genetic screens to be performed on a fully-automated, unbiased, genome-wide scale in yeast (*37*). To address the cellular roles of Aft1p I have exploited SGA technology to perform genome-wide *AFT1* synthetic lethal (SL) and synthetic dosage lethal (SDL) screens to identify non-essential yeast mutants that are sensitive to perturbations in *AFT1* under normal iron conditions. Synthetic sickness (SS) or synthetic lethality (SL) occurs when two mutants, each individually viable, cause slow growth or lethality when combined (Fig. 4). In contrast, synthetic dosage sickness (SDS) or synthetic dosage lethality (SDL) occurs when overexpression of a query gene has no notable effect on a wild-type strain but causes slow growth or lethality in a strain carrying a mutation in a gene (*43*). These described genetic interactions can be identified on a genome-wide scale in yeast for almost any gene of interest (Fig. 5). The resultant genetic interaction network can be used to identify the cellular processes that require this gene to be functional. For example, if a gene of interest, gene X,

Figure 4. Schematic illustration of synthetic genetic interactions.
A synthetic sick or synthetic lethal interaction is when the double mutant has reduced viability, either slow growth or lethality respectfully, when compared to either single mutant alone. A synthetic dosage sick or synthetic dosage lethal interaction is when overexpression of a gene of interest has no notable effect on a wild type strain but has reduced viability, either slow growth or lethality respectfully, in a strain carrying a mutation in a gene involved in the same biological pathway as the gene of interest. On the plasmids, the red arrow represents the inducible *GAL1* promoter, the blue box the query gene (2). (Used with permission)

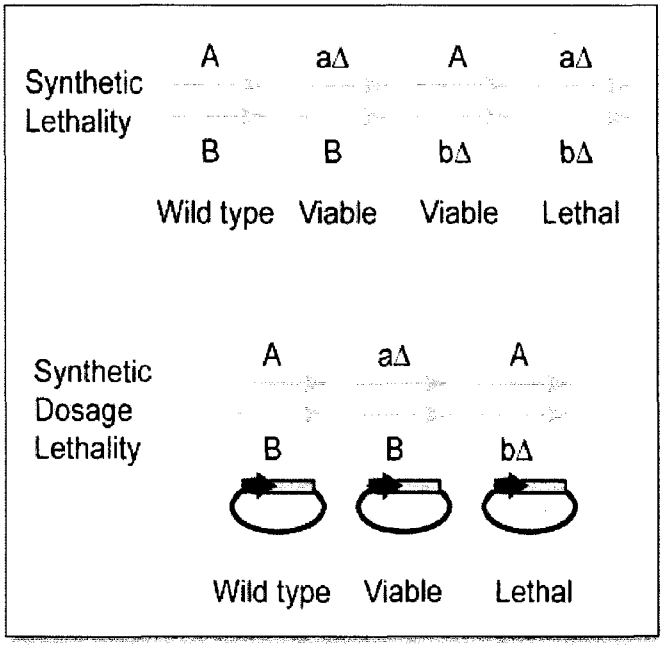
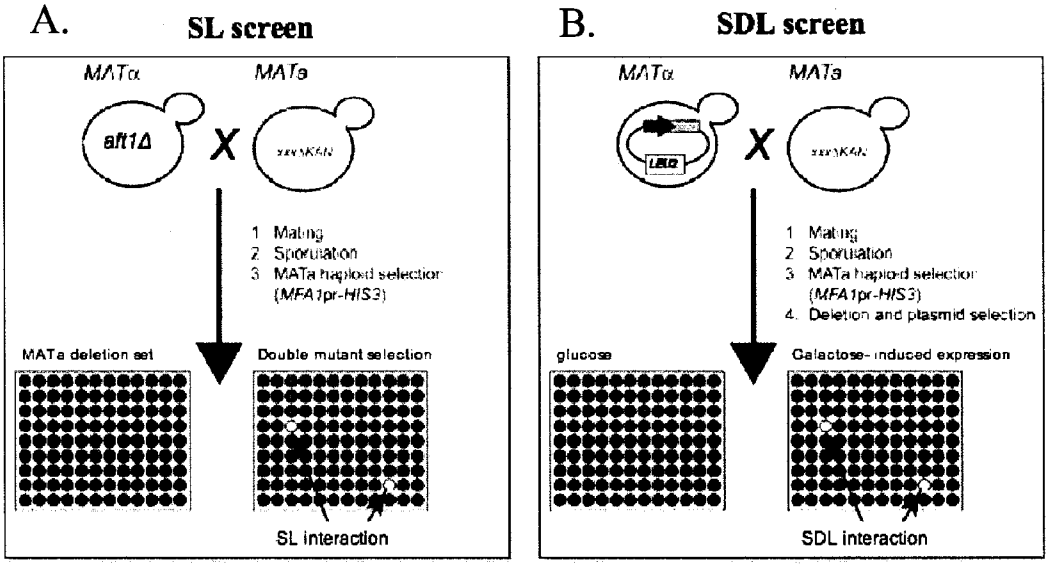

Synthetic
Lethality
A
aΔ
A
aΔ
B
B
bΔ
bΔ
Wild type
Viable
Viable
Lethal
Synthetic
Dosage
Lethality
A
aΔ
A
B
B
bΔ
Wild type
Viable
Lethal

Figure 5. Schematic illustration of systematic synthetic genetic array screening.
(A) In a synthetic lethal screen a haploid query strain with a mutation in a gene of interest (*aft1Δ*) is mated to the deletion mutant array, a collection of all haploid non-essential gene deletion mutants in yeast. After a series of replica pinning steps, double mutants are selected and assessed for a phenotype of interest (slow growth or lethality). (B) In a synthetic dosage lethal screen a haploid query strain carrying a galactose-inducible plasmid followed by a gene of interest (*AFT1*) is mated to the deletion mutant array. After a series of replica pinning steps, haploids deletion mutants containing the plasmid are selected and pinned onto galactose-containing media to induce overexpression of the *GAL1*-inducible gene. Colonies are assessed for a phenotype of interest (slow growth or lethality). (adapted from (2). Used with permission)


A.
SL screen
MATα
MATa
aft1Δ
X
1 Mating
2 Sporulation
3 MATa haploid selection
(MFA1pr-HIS3)
MATa deletion set
Double mutant selection
SL interaction
B.
SDL screen
MATα
MATa
LEU
X
1 Mating
2 Sporulation
3 MATa haploid selection
(MFA1pr-HIS3)
4. Deletion and plasmid selection
glucose
Galactose- induced expression
SDL interaction

shares genetic interactions with a large subset of genes involved in cell cycle regulation, it can be predicted that gene X somehow plays a role in cell cycle regulation as well. In this way, genetic interactions can identify functional relationships between genes (*43*).

A recent study has shown that SL and SDL screens assess different genetic relationships and these two screening techniques are non-redundant (*7, 43*). SL genetic interactions occur between genes whose gene products operate in two distinct pathways or complexes that are somehow related, whereas the basis behind SDL genetic interactions is not completely understood. Originally it was believed that both gene deletion and gene overexpression result in identical cellular phenotypes. This is described by the "balance hypothesis", a theory which suggests that an imbalance of the normal stoichiometry of a protein-protein complex, whether overexpression or deletion of an allele, is deleterious to the cell (*44*). A recent study demonstrated that this is not always true. This study found that while some overexpression phenotypes that result in slow growth or lethality can be explained by the "balance hypothesis", the majority of phenotypes are based on intolerance of the gain-of-function allele (*45*). These findings demonstrate the importance of these two individual complimentary screening techniques in probing the cellular functions of a gene of interest.

iii. Protein-protein interaction networks

Another way to functionally characterize a protein of interest is through the development of a protein-protein interaction network. Physical networks have provided an aid for researchers to understand the complex molecular relationships that determine the phenotype of a cell (*46*). As previously mentioned, the transcriptional profile of Aft1p is

well established but its protein-protein interactions are largely unknown. High-throughput two-hybrid interactions have identified an Aft1 protein-protein interaction with Yap5p (*47*), a protein involved in G_1/S-specific transcription (*48*). Other studies using affinity capture techniques have identified Aft1 protein-protein interactions with Nhp6p and Cyc8p (*49*), proteins involved in chromatin remodeling. Presently the reproducibility and the biological relevance of these Aft1p interactions have not been explored. Despite recent efforts using tandem affinity purification to process over 4000 different tagged proteins in yeast, Aft1p protein-protein interactions have not been identified by these methods (*50, 51*), likely due to the combined low expression levels of Aft1p within the cell and the lack of optimization of the purification methods for each individual protein. Therefore, a global physical interaction network for Aft1p has yet to be established, and would provide additional insight into the cellular roles that require Aft1p under normal iron conditions.

1.2 Hypothesis

While most studies have examined the transcriptional role of Aft1p under iron-deplete conditions, to date, little work has focused on the cellular roles of Aft1p under iron-replete conditions. Studies performed under normal iron conditions suggest that the biological roles of Aft1p are not limited to iron homeostasis but may be central to numerous cellular functions – many of which have yet to be explored. To gain further insight into Aft1p function, using an integrative genomics and proteomics analysis I proposed to test the hypothesis that *Aft1p impacts multiple cellular pathways under normal iron conditions.*

Chapter 2: MATERIALS AND METHODS

Reagents:

See Appendix A for list of reagents, solutions, and buffers used.

Yeast strains and media:

The yeast strains used in this study are listed in Appendix B. Deletion strains made for this study were designed using a standard PCR-mediated insertion technique (*52*) and confirmed by PCR analysis. Cells were grown in standard YPD or SD medium supplemented with amino acids (*53*), unless otherwise described.

Dot assay experiments:

Ten-fold serial dilutions (OD_{600}= 0.1, 0.01, 0.001, 0.0001) of wild type and *aft1Δ* mutant strains, grown to mid-log phase in YPD at 25°C, were plated onto media containing various compounds as indicated. Plates were incubated at 25°C for 3 days before epi-white imaging using the Molecular Imager ChemiDoc XRS System (BioRad). All dot assay experiments were repeated in duplicate using two different isolates of each strain.

Synthetic lethal screens:

Genome-wide Synthetic Lethal SGA screens were conducted in triplicate at 25°C using a RoToR HDA robot (Singer Instruments, UK) as previously described (*54*). In detail, a *MAT*α haploid query strain (either *aft1Δ::natMX4* or *rim101Δ::natMX4*), with resistance to clonNAT (nourseothricin), was mated to the *MAT*a deletion mutant array (Open Biosystems), an ordered array of ~ 4700 haploid non-essential yeast gene deletion mutants pinned in

duplicate, each linked to a kanamycin-resistance marker (*kanMX*). Selection for heterozygous diploids was achieved through pinning on medium containing clonNAT (Werner BioAgents, 5.0000) and kanamycin (Sigma, A1720). Sporulation of the resultant diploid cells, achieved through growth on medium containing reduced levels of carbon and nitrogen, produced four possible haploid meiotic spore progeny (wild type, query gene single mutant, gene-deletion single mutant, and the double mutant). Spores were transferred to synthetic medium lacking histidine, which allowed for selective germination of *MAT*a meiotic progeny (because these cells express the *MFA1pr-HIS3* reporter), and containing canavanine (Sigma, C1625) (to select for *can1Δ* mutant cells with canavanine resistance). The haploid meiotic progeny were first transferred to medium containing kanamycin, followed by medium containing clonNAT and kanamycin to specifically select for only the double-mutant meiotic progeny. Double mutants were scored for a phenotype of interest (slow growth or lethality) by visual inspection. Putative interactions (that appeared in a minimum of two out of three screens) were confirmed by tetrad analysis as previously described (*55*).

Synthetic dosage lethal screens:

Genome-wide Synthetic Dosage Lethal SGA screens were conducted in triplicate at 16°C, 25°C, and 37°C using a RoToR HDA robot (Singer Instruments, UK) as previously described (*7*). In detail, a *MAT*α haploid query strain carrying the plasmid p*GAL1-AFT1* (a galactose-inducible promoter followed by the gene *AFT1* carrying a *URA3* resistance marker), kindly provided by Michael Snyder, was mated to the *MAT*a deletion mutant array (Open Biosystems), an ordered array of ~ 4700 haploid non-essential yeast gene deletion

mutants pinned in duplicate, each linked to a kanamycin-resistance marker (*kanMX*). Diploid selection was achieved through pinning on medium containing kanamycin (Sigma, A1720) and lacking the amino acid uracil. Sporulation of the resultant diploid cells, achieved through growth on medium containing reduced levels of carbon and nitrogen, produced four possible haploid meiotic spore progeny (wild type, query carrying the plasmid, gene-deletion single mutant, and the gene-deletion single mutant carrying the plasmid). Spores were transferred to synthetic medium lacking histidine, which allowed for selective germination of *MAT*a meiotic progeny (because these cells express the *MFA1pr-HIS3* reporter), lacking uracil to select for progeny carrying the plasmid, and containing canavanine (Sigma, C1625) (to select for *can1Δ* mutant cells with canavanine resistance). The haploid meiotic progeny were first transferred to medium containing kanamycin, followed by medium containing kanamycin and lacking the amino acid uracil to specifically select for only the gene-deletion single mutant meiotic progeny carrying the plasmid. After replica pinning onto galactose media to induce overexpression of the *GAL1*-inducible *AFT1*, colonies were grown for 2 days at either 16°C, 25°C, or 37°C and scored for a phenotype of interest (slow growth or lethality) by visual inspection. Putative interactions (that appeared in a minimum of two out of three screens) were confirmed by comparing the fitness of the deletion mutant strain, in which the plasmid p*GAL1-AFT1* was reintroduced by means of traditional yeast transformation methods (*56*), to the deletion mutant strain carrying the vector control, pRS416, at all three temperatures (using streak tests or dot assays).

α-factor block and release experiments:

*MAT*a yeast strains were grown at 25°C in 50 mL of YPD to mid-log phase (OD_{600} ~0.6). Cells were harvested by centrifugation (4000 rpm, 5 min, 25°C) and resuspended in fresh YPD media (a 1 mL sample was removed for analysis by flow cytometry). The culture was arrested in G_1 phase by the addition of α-mating factor (Sigma, T6901) to a final concentration of 5 μM. After 2.5 hr of incubation and visual inspection for G_1 arrest, a 1 mL sample was removed for analysis by flow cytometry (t=0). The cultures were then split in half, harvested by centrifugation, washed once with YPD, and released from the cell cycle arrest by resuspension in YPD or YPD + 0.035% MMS (Sigma, M4016). One milliliter samples were removed for analysis by flow cytometry every 15 min for 4 hr. All α-factor block and release experiments were performed in triplicate.

Flow cytometry:

Cells were harvested by centrifugation (4000 rpm, 2 min) and fixed in 70% ethanol. Samples were then resuspended in 1 mL of 50 mM NaCitrate pH 7.4 and sonicated at low levels for 10 sec. Cells were harvested and resuspended in 1 mL of 50 mM NaCitrate pH 7.4 with 0.25 mg/mL RNAseA. After incubation at 50°C for 1 hr, 10 μL of 1.6 mg/mL propidium iodide was added and samples were incubated at RT for 30 min in the dark. Samples were then incubated with 5 μL of 0.5 mg/mL proteinase K at 50°C for 1 hr. Samples were analyzed immediately using a flow cytometer (Beckman-Coulter, FC500) or stored at 4°C until analysis.

Aft1p-TAP protein purification and identification:

One-step affinity purification of Aft1p-TAP was performed, in duplicate, using IgG coated magnetic beads. Cells from 4 L of mid-log phase culture (OD_{600} ~ 0.6-0.8) grown in YPD at 25°C were collected by centrifugation (5000 rpm, 10 min, 4°C), washed in 80 mL Lysis Buffer (20 mM HEPES pH 7.4, 0.1% Tween-20, 2 mM $MgCl_2$, 300 mM NaCl, Protease Inhibitor Cocktail (Sigma, P-8215), resuspended in 5 mL Lysis Buffer and transferred in 1 mL aliquots to 2 mL screw-cap tubes. Cells were again harvested by centrifugation and resuspended in 500 µL Lysis Buffer plus an equal volume of acid washed glass beads (Fisher Scientific, 35-535) and lysed through vortexing (10 x 1 min vortex with 1 min incubation on ice in between vortexing). The soluble whole cell extract (WCE) was isolated by centrifugation (2x 10 min, 14000 rpm, 4°C) and 350 mg of WCE was incubated (overnight, 4°C, with rotation) with 50 µL of magnetic dynabeads (Dynal, Invitrogen 143-01) cross-linked with rabbit IgG (Chemicon, PP64) as per manufacturer's instructions. Dynabeads were collected with a magnet and washed ten times with 500 µL cold Lysis Buffer. The dynabeads were resuspended in 30 µL of 1 x loading buffer (50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol) and proteins were eluted from the beads using heat (65°C, 10 min). Dynabeads were collected with a magnet and sample was transferred to a new 1.5 mL tube. Samples were boiled for 5 min and 25 µL were resolved on a 4-12% pre-cast polyacrylamide gradient gel (Invitrogen, NP0321BOX) in 1X MOPS buffer. Proteins were visualized by coomassie staining.

Immunoblot analysis:

One-step affinity purification of TAP-tagged strains was performed using IgG coated magnetic beads as described above, with the following changes: immunoblot analysis of the Aft1p-TAP tagged protein purification was performed in duplicate using a 4 L starting culture, whereas immunoblot analysis of the co-immunopurification of Aft1p and Mcm3p was performed in triplicate using a 1 L starting culture of Aft1p-Myc, Mcm3p-TAP, and Aft1p-Myc Mcm3p-TAP. Samples were boiled for 5 min and 5 μL were resolved on a 4-12% pre-cast polyacrylamide gradient gel (Invitrogen, NP0321BOX) in 1X MOPS buffer. Proteins were electrophoretically transferred onto nitrocellulose membranes using semi-dry transfer (BioRad). The membranes were blocked in Phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.1% Tween 20 (PBS-T), for 1 hr at room temperature with shaking, or at 4°C overnight. Primary and secondary antibodies were diluted in the blocking solution and incubated with the membranes at room temperature for 1 hr with shaking. After incubation with each antibody, the membranes were washed with shaking in triplicate with PBS-T for 10 min at room temperature. The primary antibodies used in these studies were: α-TAP (Open Biosystems, CAB1001) (1:5000) and α-Myc (Roche, 11667149) (1:10000). Secondary antibodies used in these studies were peroxidase-conjugated goat α-rabbit IgG (Chemicon, AP307P) (1:5000) and peroxidase-conjugated goat α-mouse IgG (BioRad, 170-6516) (1:3000). Membranes were developed using ECL Plus Western Blotting Detection System (Amersham BioSciences, RPN2135) according to the manufacturer's instructions.

Mass spectrometry identification of proteins:

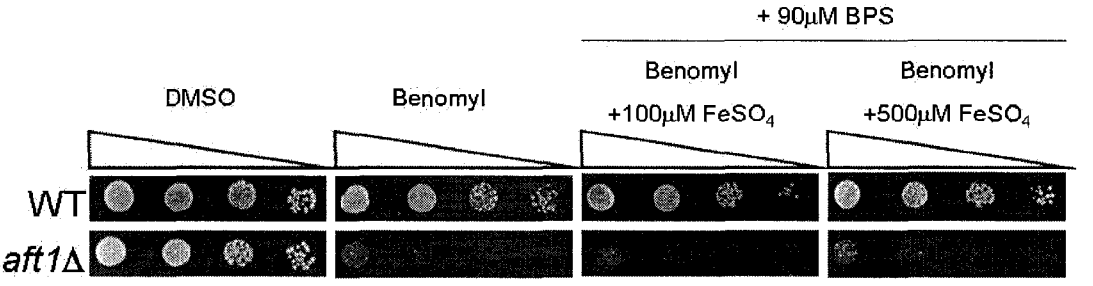
Gel bands, encompassing the entire wild type and Aft1p-TAP tagged sample lanes, were excised and subjected to in-gel trypsin digestion following standard protocols(*57*). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed by Jean-Philippe Lambert from the laboratory of Dr. Daniel Figeys using an LTQ quadrupole ion-trap mass spectrometer (Thermo-Electron, Waltham, MA) as previously described(*58*). MS/MS data were analyzed and matched to *S. cerevisiae* protein sequences in the NCBI database (nr) using the Mascot database search engine (Matrix Science Inc, Boston, MA).

Chapter 3: RESULTS

Iron-independent role of AFT1 in cellular response to benomyl

A recent study has demonstrated through unbiased, genome-wide screens that Aft1p is required for faithful chromosome transmission (*7*). As many mutants with defects in chromosome stability also have increased sensitivity to the microtubule-destabilizing drug Benomyl (*59*), I asked whether *aft1Δ* mutants are also hypersensitive to Benomyl treatment. Analysis of 10-fold serial dilutions in a spot assay on plates either containing no drug (DMSO) or 10 μg/mL Benomyl, showed that *aft1Δ* mutant cells are hypersensitive to Benomyl treatment compared to wild type control cells (Fig. 6). A recent study demonstrated that *aft1Δ* mutant cell hypersensitivity to the drug hydroxyurea, an inhibitor of dNTP synthesis, was suppressible by increasing levels of iron in the growth medium (*36*). Therefore I was interested in determining whether *aft1Δ* mutant cell hypersensitivity to Benomyl treatment could also be rescued by increased levels of iron. To investigate this, the spot assay was repeated on plates containing media with restricted levels of iron, through the addition of an iron chelator bathophenantroline-disulphonate (BPS) and various levels of $FeSO_4$ (10, 25, 50, 100, 200, 500, and 1000μM) (restricted iron conditions were based on previous experiments (*60*)). The slow-growth phenotype of *aft1Δ* mutants in response to Benomyl treatment was not rescued by increasing levels of iron in the growth medium (Fig. 6), suggesting that the cellular role of Aft1p in chromosome transmission fidelity is independent of cellular iron levels.

Figure 6. Increased iron levels do not rescue *aft1Δ* hypersensitivity to the microtubule-destabilizing drug, Benomyl. *MAT*a wild type (YKB1032) and *aft1Δ::kanMX6* (YKB673) strains were grown in rich medium (YPD) at 25°C to an OD_{600} of 0.6. Cultures were diluted to an OD_{600} of 0.1, and 10-fold serial dilutions were spotted onto rich medium containing 10 μg/mL benomyl resuspended in DMSO, 90 μM bathophenantroline-disulphonate (BPS, an iron chelator), and the indicated concentrations of $FeSO_4$. Plates were incubated at 25°C for 3 days. A representative of one of three experiments (which displayed similar results) is shown with DMSO used as a control.


+ 90μM BPS
DMSO
Benomyl
Benomyl
+100μM FeSO4
Benomyl
+500μM FeSO4
WT
aft1Δ

Synthetic lethal genetic interaction map indicates Aft1p impacts a diverse range of cellular processes under normal iron conditions

Both Measday et al. 2005 and my results suggest that Aft1p has an iron-independent role in chromosome transmission fidelity; hence I was interested in determining whether Aft1p has additional cellular roles under normal iron conditions. To elucidate the cellular roles of Aft1p, genome-wide synthetic lethal SGA screens were utilized to identify non-essential yeast mutants that are sensitive to the deletion of *AFT1* under normal iron conditions. The SL-SGA screen was performed in triplicate using SGA methodology (*54*) and putative genetic interactions that resulted in inviability (synthetic lethality) or reduced fitness (synthetic sickness) a minimum of two out of three times, were confirmed by tetrad analysis (Table 2). The resulting confirmed data set contains 45 genetic interactions of which 22% (10/45) were synthetic lethal interactions, while the remainders were synthetic sick interactions.

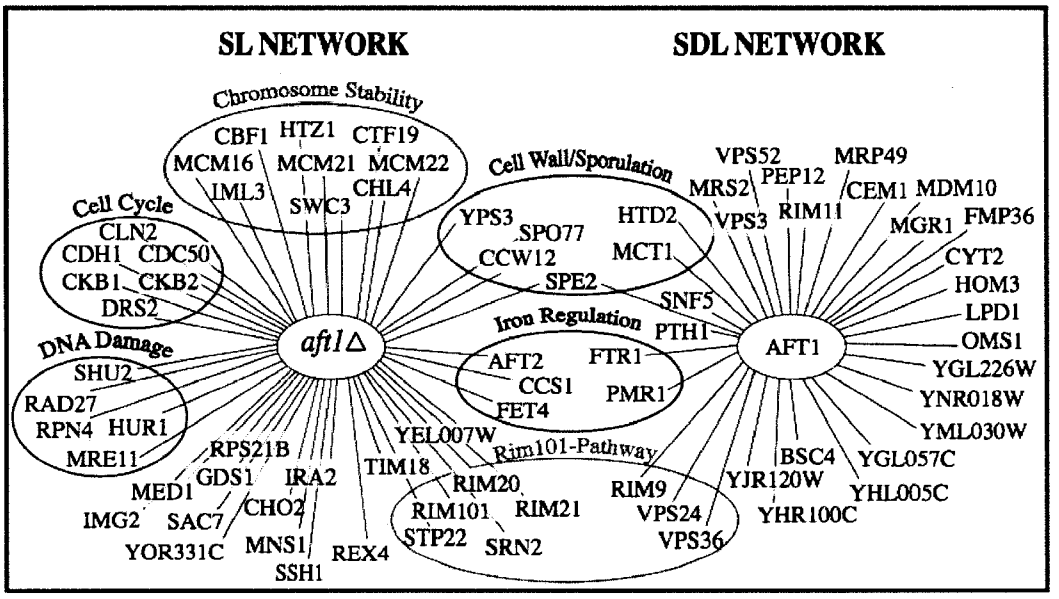
Given that genetic interactions predict functional relationships (*54*), I anticipated that many genes in the *AFT1* genetic interaction map would participate in processes to which Aft1p has been previously linked. As expected, many of the genes in the *AFT1* genetic interaction network had been previously implicated in iron regulation and chromosome stability as determined by their GO annotations (Fig. 7), suggesting that the genetic map is of high quality. In addition to the well-characterized roles for Aft1p, the genetic interaction map identified genes implicated in a wide variety of functions, including the *Rim101* pH response pathway, cell cycle regulation, DNA damage response, and cell wall assembly and sporulation. Overall, this network strongly suggests that although Aft1p has an integral role

Table 2. *AFT1* Synthetic Lethal Genetic Interactions

Deletion strain ORF	Standard name	Interaction type (SS or SL*)	Cellular function**
YAL011W	*SWC3*	SS	component of Swr1p complex that incorporates Htz1p into chromatin
YAL026C	*DRS2*	SS	integral membrane Ca(2+)-ATPase
YBR107C	*IML3*	SS	protein with a role in kinetochore function
YBR283C	*SSH1*	SS	subunit of the Ssh1 translocon complex, involved in protein translocation
YCL008C	*STP22*	SL	component of the ESCRT-I complex
YCR071C	*IMG2*	SL	mitochondrial ribosomal protein of the small subunit
YCR094W	*CDC50*	SL	endosomal protein that regulates cell polarity
YDL020C	*RPN4*	SS	transcription factor that stimulates expression of proteasome genes
YDR078C	*SHU2*	SS	protein involved in mutation suppression, important for error-free repair of DNA lesions
YDR254W	*CHL4*	SS	outer kinetochore protein required for chromosome stability
YDR318W	*MCM21*	SS	protein involved in minichromosome maintenance
YDR389W	*SAC7*	SS	GTPase activating protein (GAP) for Rho1p, involved in signaling to the actin cytoskeleton
YEL007W		SS	protein of unknown function
YGL003C	*CDH1*	SS	cell-cycle regulated activator of the anaphase-promoting complex/cyclosome (APC/C)
YGL019W	*CKB1*	SS	a Ser/Thr protein kinase with roles in cell growth and proliferation
YGL168W	*HUR1*	SL	protein required for hydroxyurea resistance, has a possible role in DNA replication
YGR157W	*CHO2*	SS	phosphatidylethanolamine methyltransferase (PEMT)
YHL027W	*RIM101*	SS	transcriptional repressor involved in the response to pH
YJL136C	*RPS21B*	SS	protein component of the small (40S) ribosomal subunit
YJR060W	*CBF1*	SS	helix-loop-helix protein that binds to the motif CACRTG, targets Isw1p to DNA
YJR131W	*MNS1*	SS	alpha-1,2-mannosidase involved in ER quality control
YJR135C	*MCM22*	SS	protein involved in minichromosome maintenance, component of the kinetochore
YKL113C	*RAD27*	SS	5' to 3' exonuclease, required for Okazaki fragment processing
YLR110C	*CCW12*	SS	cell wall protein
YLR119W	*SRN2*	SS	component of ESCRT-I complex
YLR121C	*YPS3*	SS	aspartic protease, attached to the plasma membrane via GPI anchor
YLR341W	*SPO77*	SS	meiosis-specific protein required for spore wall formation during sporulation
YMR038C	*CCS1*	SS	copper chaperone, involved in oxidative stress protection
YMR224C	*MRE11*	SS	subunit of a complex with Rad50p and Xrs2p that functions in repair of DNA double-strand breaks
YMR319C	*FET4*	SL	low-affinity Fe(II) transporter of the plasma membrane
YNL294C	*RIM21*	SL	protein involved in the response to alkaline pH
YOL012C	*HTZ1*	SS	histone variant H2AZ, exchanged for histone H2A in nucleosomes by the SWR1 complex
YOL052C	*SPE2*	SS	S-adenosylmethionine decarboxylase, required for biosynthesis of spermidine and spermine
YOL080C	*REX4*	SL	putative RNA exonuclease possibly involved in pre-rRNA processing and ribosome assembly
YOL081W	*IRA2*	SS	GTPase-activating protein that negatively regulates RAS
YOR039W	*CKB2*	SS	a Ser/Thr protein kinase with roles in cell growth and proliferation
YOR275C	*RIM20*	SS	protein involved in proteolytic activation of Rim101p in response to alkaline pH
YOR297C	*TIM18*	SS	component of the mitochondrial Tim54p-Tim22p complex
YOR331C		SL	protein of unknown function
YOR355W	*GDS1*	SL	protein of unknown function, required for growth on glycerol as a carbon source
YPL018W	*CTF19*	SS	outer kinetochore protein required for accurate mitotic chromosome segregation
YPL202C	*AFT2*	SL	iron-regulated transcriptional activator
YPL256C	*CLN2*	SS	G1 cyclin involved in regulation of the cell cycle
YPR046W	*MCM16*	SS	protein involved in kinetochore-microtubule mediated chromosome segregation
YPR070W	*MED1*	SS	subunit of RNA polymerase II mediator complex

*SS=synthetic sick, SL=synthetic lethal. **Adapted from Gene Ontology Annotations/Biological Processes listed in Saccharomyces Genome Database (www.yeastgenome.org)

Figure 7. *AFT1* function is required for multiple cellular functions under normal iron conditions. Synthetic genetic interactions were identified for the gene encoding the transcription factor *AFT1* on a genome-wide scale using SL- and SDL-SGA technology. Circles are used to highlight enrichment for genes of a certain biological process (as determined by GO process) in the data. Only biological processes that are important to this study are circled. Lines indicate genetic interactions, either synthetic sick or synthetic lethal for the SL-SGA screen or synthetic dosage sick or synthetic dosage lethal for the SDL-SGA screen. Only one genetic interaction is shared (gene highlighted in red) between these two screens.


SL NETWORK
SDL NETWORK
Chromosome Stability
CBF1
HTZ1
CTF19
MCM16
MCM21
MCM22
IML3
SWC3
CHL4
Cell Cycle
CLN2
CDH1
CDC50
CKB1
CKB2
DRS2
DNA Damage
SHU2
RAD27
RPN4
HUR1
MRE11
aft1Δ
Cell Wall/Sporulation
YPS3
SPO77
CCW12
SPE2
Iron Regulation
AFT2
CCS1
FET4
FTR1
PMR1
PTH1
AFT1
HTD2
MCT1
SNF5
VPS52
MRP49
MRS2
PEP12
VPS3
RIM11
CEM1
MDM10
MGR1
FMP36
CYT2
HOM3
LPD1
OMS1
YGL226W
YNR018W
YML030W
YGL057C
YHL005C
YHR100C
YJR120W
BSC4
RPS21B
GDS1
IRA2
MED1
IMG2
SAC7
CHO2
YOR331C
MNS1
SSH1
REX4
TIM18
YEL007W
Rim101-Pathway
RIM20
RIM101
RIM21
STP22
SRN2
RIM9
VPS24
VPS36

in iron homeostasis under low iron conditions, there are additional pathways that are impacted by Aft1p under normal iron conditions.

Synthetic dosage lethal genetic interaction map suggests that Aft1p may function in parallel with Rim101p

To further probe the cellular processes potentially impacted by Aft1p, a complimentary technique called synthetic dosage lethal (SDL) SGA screening, was utilized to identify non-essential yeast mutants that are sensitive to the overexpression of *AFT1* under normal iron conditions. The SDL-SGA screen was performed in triplicate, at three different temperatures (16°C, 25°C, and 37°C), using SGA methodology (*7*) and putative genetic interactions that resulted in inviability (synthetic dosage lethality) or reduced fitness (synthetic dosage sickness) in a minimum of two out of three screens, were confirmed by streak test analysis as described in the Materials and Methods section (Table 3). The resulting confirmed data set contains 32 genetic interactions of which 3% (1/32) were synthetic dosage lethal interactions, while the remainders were synthetic dosage sick interactions.

A recent study has shown that SL and SDL screens assess different genetic relationships largely identifying distinct sets of interactions (*7*, *43*). Though these two screening techniques are non-redundant, previous studies have shown that genes identified in both screens or genes implicated in the same cellular processes but that were identified separately in either the SL or SDL screen can provide powerful insights into the query strain's function (*7*). Therefore I integrated the results from both of the screens to develop an *AFT1* genetic interaction network (Fig. 7). Only *SPE2*, a gene involved in the biosynthesis

Table 3. *AFT1* Synthetic Dosage Lethal Genetic Interactions

Deletion Strain			
ORF	Standard name	Interaction Type (SDS or SDL*), (temperature °C)	Cellular Function**
YAL010C	*MDM10*	SDS, (16)	subunit of the mitochondrial sorting and assembly machinery
YBR289W	*SNF5*	SDS, (25)	one of 11 subunits of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation
YCL044C	*MGR1*	SDS, (25)	subunit of mitochondrial inner membrane I-AAA protease complex
YDR316W	*OMS1*	SDS, (16, 25)	protein integral to the mitochondrial membrane
YDR484W	*VPS52*	SDS, (25)	component of the GARP (Golgi-associated retrograde protein) complex
YDR493W	*FMP36*	SDS, (25)	protein localized to the mitochondria
YDR495C	*VPS3*	SDS, (16, 37)	cytoplasmic protein required for the sorting and processing of vacuolar proteins
YER052C	*HOM3*	SDS, (16, 37)	aspartate kinase, cytoplasmic enzyme
YER061C	*CEM1*	SDS, (16, 25, 37)	mitochondrial beta-keto-acyl synthase with possible role in fatty acid synthesis
YER145C	*FTR1*	SDS, (25)	high affinity iron permease involved in the transport of iron across the plasma membrane
YFL018C	*LPD1*	SDS, (16)	dihydrolipoamide dehydrogenase
YGL057C		SDS, (25)	function unknown
YGL167C	*PMR1*	SDS, (16, 25)	high affinity Ca2+/Mn2+ P-type ATPase required for Ca2+ and Mn2+ transport into Golgi
YGL226W		SDS, (25)	function unknown
YHL005C		SDL, (25, 37)	function unknown
YHR067W	*HTD2*	SDS, (16, 25)	mitochondrial 3-hydroxyacyl-thioester dehydratase involved in fatty acid biosynthesis
YHR100C		SDS, (25)	function unknown
YHR189W	*PTH1*	SDS, (16, 25, 37)	one of two mitochondrially-localized peptidyl t-RNA hydrolases
YJR120W		SDS, (25)	function unknown
YKL041W	*VPS24*	SDS, (25)	one of four subunits of the endosomal sorting complex required for transport III
YKL087C	*CYT2*	SDS, (25)	cytochrome c1 heme lyase, links heme to apocytochrome c1
YKL167C	*MRP49*	SDS, (25, 37)	mitochondrial ribosomal protein of the large subunit
YLR417W	*VPS36*	SDS, (25, 37)	component of the ESCRT-II complex
YML030W		SDS, (16, 25, 37)	function unknown
YMR063W	*RIM9*	SDS, (25, 37)	involved in the proteolytic activation of Rim101p in response to alkaline pH
YMR139W	*RIM11*	SDS, (25)	protein kinase required for signal transduction during entry into meiosis
YNL269W	*BSC4*	SDS, (25, 37)	function unknown
YNR018W		SDS, (25)	function unknown
YOL052C	*SPE2*	SDS, (25)	S-adenosylmethionine decarboxylase, required for biosynthesis of spermidine and spermine
YOR036W	*PEP12*	SDS, (16)	target membrane receptor (t-SNARE) for vesicular intermediates traveling between the Golgi and the vacuole
YOR221C	*MCT1*	SDS, (25)	predicted malonyl-CoA:ACP transferase, mitochondrial fatty acid synthase
YOR334W	*MRS2*	SDS, (25)	mitochondrial inner membrane Mg(2+) channel

*SDS=synthetic dosage sick, SDL=synthetic dosage lethal. **Adapted from Gene Ontology Annotations/Biological Processes listed in Saccharomyces Genome Database (www.yeastgenome.org)

of spermidine and spermine (*61*), was identified in both the SL and SDL screens. As expected both screens identified genes implicated in iron regulation. In addition, both screens identified genes involved in the Rim101 pH response pathway, cell wall integrity, and sporulation, which strongly suggests that Aft1p may be implicated in these cellular processes.

Aft1p and Rim101p, a potentially shared role in cell wall assembly

The most striking observation I made from the *AFT1* genetic interaction map is that seven of the genes identified participate in the Rim101 pH response pathway (*31*). This represents an enrichment for 44% (7/16) of the genes that have been previously implicated to participate in this cellular pathway (Table 4). The Rim101 pathway plays a role in the transcriptional response to alkaline pH (*31*), as well as cell wall assembly (*32*) and sporulation (*28*). The *AFT1* genetic screen also identified genes involved in all three of these cellular processes, further suggesting a connection between Aft1p and Rim101p cellular function. Furthermore, studies have shown that *aft1Δ* mutants display sporulation defects (*62*) as well as hypersensitivity to alkaline pH (*30*). The identification of numerous genes implicated in cell wall assembly in my *AFT1* genetic interaction network suggest that Aft1p may also have a role in cell wall assembly. As many mutants with defects in cell wall structure display hypersensitivity to sodium-dodecyl sulfate (SDS) (which interferes with membrane synthesis), caffeine (which activates a map kinase cascade altering cell wall assembly), and calcofluor (which binds to cell wall chitin) in the growth medium (*32*), I asked whether *aft1Δ* mutants are also hypersensitive to treatment with these compounds. Analysis of 10-fold serial dilutions in a spot assay on plates either containing no drug (YPD),

Table 4. Genes involved in the Rim101 pH-responsive pathway identified in *AFT1* genetic interaction screens

Gene *	Screen Type (SL or SDL**)
Encodes proteins involved in initiating proteolytic activation of Rim101p in response to alkaline p H	
RIM21	SL
RIM9	SDL
RIM8	none
Encodes proteins that form the ESCRT-III complex; accumulates Vps20p and Snf7 at the endosomal membrane in response to alkaline p H	
VPS24	SDL
DID4	none
VPS4	none
VPS20	none
SNF7	none
Encodes proteins that form the ESCRT-I complex; ubiquitin-dependent sorting of proteins into the endosome	
STP22	SL
VPS28	none
Encodes proteins that form the ESCRT-II complex; ubiquitin-dependent sorting of proteins into the endosome	
VPS36	SDL
VPS25	none
SNF8	none
Encodes proteins involved in executing proteolytic activation of Rim101p in response to alkaline p H	
RIM20	SL
RIM13	none
Encodes a transcriptional repressor involved in response to alkaline pH; activated by proteolytic processing	
RIM101	SL

*genes involved in the Rim101 pH-response pathways as indicated in (31)
**SL=synthetic lethal SGA screen, SDL=synthetic dosage lethal SGA screen

0.002% SDS, 4 mM caffeine, or 5 μg/mL calcofluor, showed that *aft1Δ* mutant cells are hypersensitive to all three compounds in the growth media compared to wild type control cells (Fig. 8). This suggests that Aft1p may have a functional role in cell wall assembly.

Aft1p has unique cellular roles under normal iron conditions compared to Rim101p

The *AFT1* genetic interaction network suggests that Aft1p functions in parallel with the Rim101 pathway. Additionally, our results demonstrate that *aft1Δ* mutants share similar phenotypes with other genes involved in the cellular pathways governed by the Rim101 pathway, including alkaline pH response, cell wall assembly, and sporulation. Furthermore, several microarray studies have shown that upon alkaline pH treatment, expression levels of genes involved in iron acquisition and utilization are increased (*27*) (*28, 29*). All of these results strongly indicate that Aft1p and Rim101p may have parallel or shared cellular functions. To explore this question, a genome-wide synthetic lethal (SL) SGA screen was performed in triplicate with a *rim101Δ* mutant strain using SGA methodology (*54*). Putative genetic interactions that resulted in inviability (synthetic lethality) or reduced fitness (synthetic sickness) a minimum of two out of three times, were confirmed by tetrad analysis (Table 5). The resulting confirmed data set contains 27 genetic interactions of which 22% (6/27) were synthetic lethal interactions, while the remainders were synthetic sick interactions. Despite numerous shared phenotypes, remarkably, *aft1Δ* and *rim101Δ* only share five common synthetic genetic interactions with *IMG2*, *MED1*, *SAC7*, *YOR331C*, and *MRE11* (Fig. 9, highlighted in red). However both screens identified genes implicated in iron regulation, cell wall assembly, and sporulation, further confirming that both Aft1p and Rim101p participate in these cellular functions. Interestingly, the *RIM101* screen also

Figure 8. *aft1Δ* mutants are hypersensitive to SDS, caffeine, and calcoflour in the growth medium. Two different isolates of *MAT*a wild type (YKB1032) and *aft1Δ::kanMX6* (YKB673) strains were grown in rich medium (YPD) at 25°C to an OD_{600} of 0.6. Cultures were diluted to an OD_{600} of 0.1, and 10-fold serial dilutions were spotted onto rich medium containing either 0.002% sodium dodecyl sulfate (SDS), 4 mM caffeine, or 5 μg/mL calcofluor. All three compounds are known to cause a growth fitness defect in cell wall assembly mutants. Plates were incubated at 25°C for 3 days. A representative of one of three experiments (which displayed similar results) is shown.

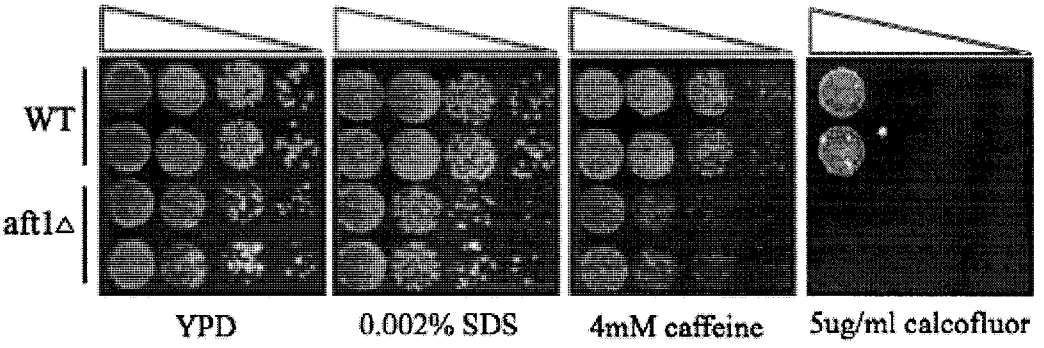
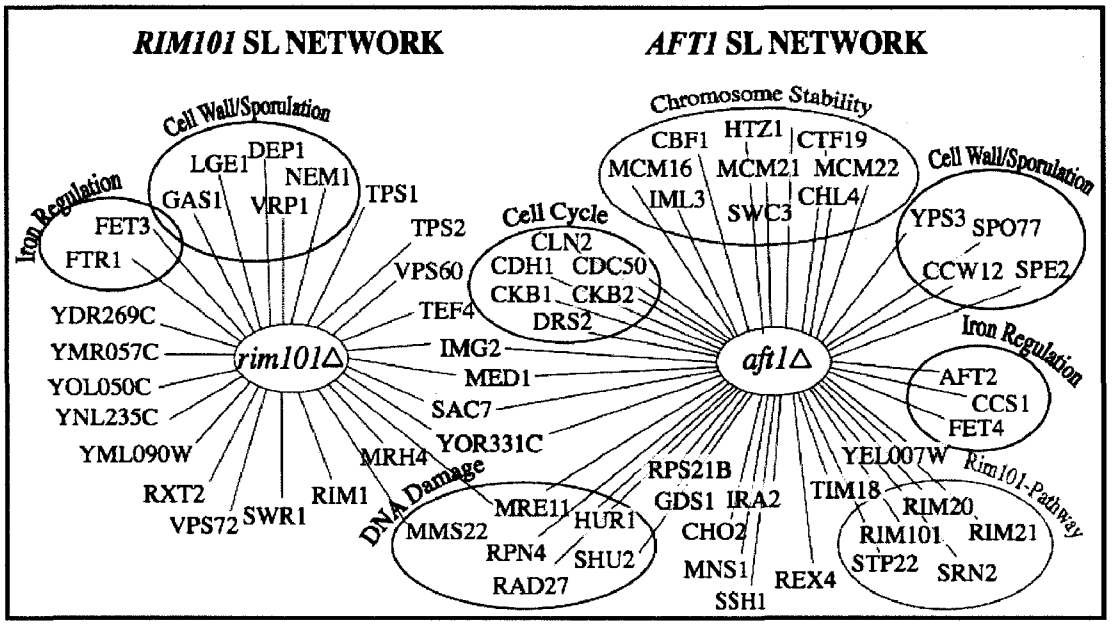

WT
aft1Δ
YPD
0.002% SDS
4mM caffeine
5ug/ml calcofluor

Table 5. *RIM101* Synthetic Lethal Genetic Interactions

Deletion Strain ORF	Standard name	Interaction Type (SS or SL*)	Cellular Function**
YAL013W	*DEP1*	SS	transcriptional modulator, regulates genes involved in phospholipid biosynthesis
YBR095C	*RXT2*	SS	protein involved in cell fusion and invasive growth
YBR126C	*TPS1*	SL	synthase subunit of trehalose-6-phosphate synthatase/phosphatase complex
YCR028C-A	*RIM1*	SS	single-stranded DNA-binding protein essential for mitochondrial genome maintenance
YCR071C	*IMG2*	SL	mitochondrial ribosomal protein of the small subunit
YDR074W	*TPS2*	SS	phosphatase subunit of the trehalose-6-phoshate synthase/phosphatase complex
YDR269C		SS	function unknown
YDR334W	*SWR1*	SS	Swi2/Snf2-related ATPase, component of SWR1 complex
YDR389W	*SAC7*	SS	GTPase activating protein (GAP) for Rho1p
YDR485C	*VPS72*	SS	component of Swr1p complex that incorporates Htz1p into chromatin
YDR486C	*VPS60*	SS	cytoplasmic and vacuolar membrane protein involved in late endosome to vacuole transport
YER145C	*FTR1*	SS	high affinity iron permease involved in the transport of iron across the plasma membrane
YGL064C	*MRH4*	SL	mitochondrial RNA helicase, plays an essential role in mitochondrial function
YHR004C	*NEM1*	SS	protein of the nuclear envelope, required for normal sporulation
YKL081W	*TEF4*	SS	translation elongation factor EF-1 gamma
YLR320W	*MMS22*	SS	protein involved in resistance to ionizing radiation
YLR337C	*VRP1*	SS	proline-rich, actin-associated protein involved in cytoskeletal organization
YML090W		SS	function unknown
YMR057C		SS	function unknown
YMR058W	*FET3*	SS	ferro-O2-oxidoreductase required for high-affinity iron uptake
YMR224C	*MRE11*	SS	subunit of a complex with Rad50p and Xrs2p that functions in repair of DNA double-strand breaks
YMR307W	*GAS1*	SS	beta-1,3-glucanosyltransferase, required for cell wall assembly
YNL235C		SL	function unknown
YOL050C		SL	function unknown
YOR331C		SL	function unknown
YPL055C	*LGE1*	SS	null mutant forms abnormally large cells
YPR070W	*MED1*	SS	subunit of RNA polymerase II mediator complex

*SS=synthetic sick, SL=synthetic lethal. **Adapted from Gene Ontology Annotations/Biological Processes listed in Saccharomyces Genome Database (www.yeastgenome.org)

Figure 9. *RIM101* and *AFT1* have unique synthetic genetic interaction maps.
Synthetic genetic interactions were identified for the genes encoding the transcription factors *RIM101* and *AFT1* on a genome-wide scale using SL-SGA technology. Circles are used to highlight enrichment for genes of a certain biological process (as determined by GO process) in the data. Only biological processes that are important to this study are circled. Lines indicate genetic interactions, either synthetic sick or synthetic lethal. Only five genetic interactions are shared (genes highlighted in red) between these two genes.


RIM101 SL NETWORK
AFT1 SL NETWORK
Cell Wall/Sporulation
LGE1
DEP1
NEM1
GAS1
VRP1
TPS1
Iron Regulation
FET3
FTR1
TPS2
VPS60
TEF4
YDR269C
YMR057C
YOL050C
YNL235C
YML090W
RXT2
VPS72
SWR1
RIM1
rim101Δ
IMG2
MED1
SAC7
YOR331C
MRH4
DNA Damage
MRE11
MMS22
HUR1
RPN4
SHU2
RAD27
Cell Cycle
CLN2
CDH1
CDC50
CKB1
CKB2
DRS2
Chromosome Stability
CBF1
HTZ1
CTF19
MCM16
MCM21
MCM22
IML3
SWC3
CHL4
afl1Δ
RPS21B
GDS1
IRA2
CHO2
MNS1
SSH1
REX4
Cell Wall/Sporulation
YPS3
SPO77
CCW12
SPE2
Iron Regulation
AFT2
CCS1
FET4
YEL007W
TIM18
Rim101-Pathway
RIM20
RIM101
RIM21
STP22
SRN2

identified genes involved in DNA damage repair including *MMS22* and *MRE11*, suggesting that similar to Aft1p, Rim101p may also be implicated in DNA damage repair. The other cellular roles of Aft1p, such as chromosome stability and cell cycle regulation, were not identified in the *rim101Δ* SL-SGA screen suggesting that these cellular functions are unique to Aft1p.

Aft1p has additional biological functions that are likely independent of cellular iron levels

To further investigate whether the additional roles identified for Aft1p are independent of cellular iron levels, I asked whether *AFT2*, a gene that encodes for the paralog of Aft1p (*24*), or *FET3*, an iron-regulon gene that encodes for a member of the high-affinity iron transport system (*15*), share genetic interactions with any of the genes identified in the *AFT1* SL-SGA screen. SL-SGA mini-screens were performed in which the 45 deletion mutants that were identified in the *aft1Δ* SL-SGA screen were directly tested with *aft2Δ* and *fet3Δ* mutant query strains using SGA methodology (*54*). Putative genetic interactions that resulted in inviability (synthetic lethality) or reduced fitness (synthetic sickness) a minimum of two out of three times, were confirmed by tetrad analysis (Table 6). The resulting confirmed data set for the *AFT2* SL-SGA mini-screen contains 3 genetic interactions of which 7% (3/45) are shared with the *AFT1* SL-SGA screen. The resulting confirmed data set for the *FET3* SL-SGA mini-screen contains 8 genetic interactions of which 18% (8/45) are shared with the *AFT1* SL-SGA screen. For both *AFT2* and *FET3* the identified genetic interactors are involved in either iron regulation, sporulation, or the Rim101 pathway, suggesting that the cellular roles for Aft1p in chromosome stability, cell cycle regulation, and DNA damage repair are likely not dependent on cellular iron levels.

Table 6. *AFT1* Synthetic Lethal Genetic Interactions Shared with *AFT2* and *FET 3*

Deletion Strain		Interaction Type (SS or SL*)			Cellular function***
ORF	Standard name	*AFT1***	*AFT2***	*FET3***	
YPL202C	*AFT2*	SL	N/A		iron-regulated transcriptional activator
YOL052C	*SPE2*	SS	SS	SS	S-adenosylmethionine decarboxylase, required for biosynthesis of spermidine and spermine
YEL007W		SS	SS	SS	protein of unknown function
YPR070W	*MED1*	SS	SS		subunit of RNA polymerase II mediator complex
YHL027W	*RIM101*	SS		SL	transcriptional repressor involved in the response to pH
YCL008C	*STP22*	SL		SL	component of the ESCRT-I complex
YMR319C	*FET4*	SL		SL	low-affinity Fe(II) transporter of the plasma membrane
YOR275C	*RIM20*	SS		SL	protein involved in proteolytic activation of Rim101p in response to alkaline pH
YNL294C	*RIM21*	SL		SS	protein involved in the response to alkaline pH
YMR038C	*CCS1*	SS		SS	copper chaperone, involved in oxidative stress protection

*SS=synthetic sick, SL=synthetic lethal

**Query gene deletion mutant

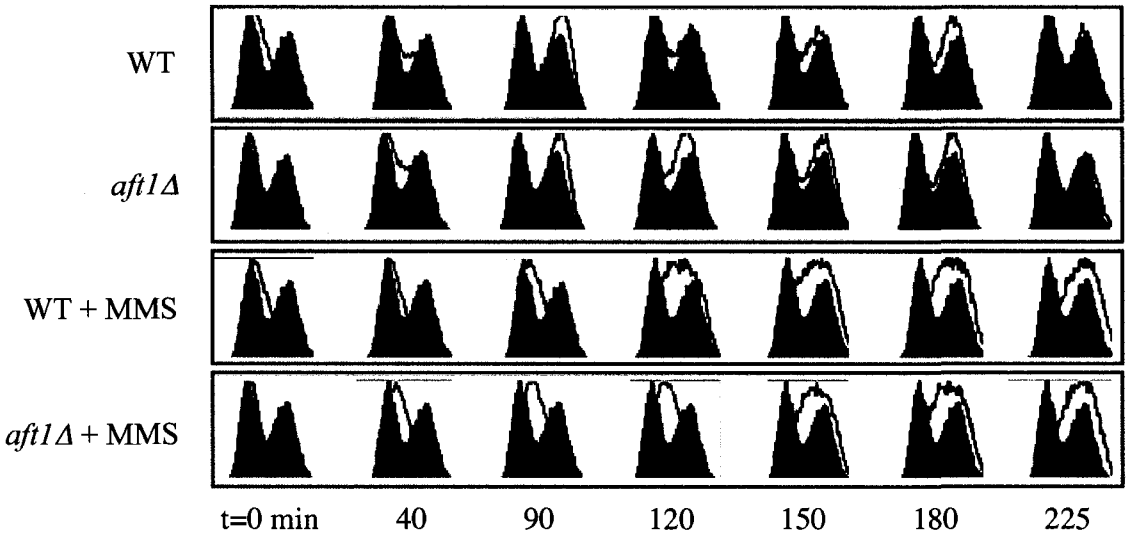
***Adapted from Gene Ontology Annotations/Biological Processes listed in Saccharomyces Genome Database (www.yeastgenome.org)

An undefined role for Aft1p in DNA damage repair

Interestingly, the *AFT1* genetic interaction map suggests that Aft1p is involved in two cellular processes that are not conventionally associated to this protein, cell cycle regulation and DNA damage repair (Fig. 7). Although there has been some indication of the requirement for Aft1p function within these pathways, the molecular mechanisms have yet to be explored. For example, one study demonstrated that *aft1Δ* mutant cells are significantly larger than wild type cells suggesting a delay through the G_1/S phase transition in the cell cycle (*8*). Another study demonstrated that *aft1Δ* mutant cells are hypersensitive to hydroxyurea, a drug that inhibits dNTP synthesis and replication (*36*). To determine if Aft1p is required for proper progression through the cell cycle I performed a series of alpha factor block and release flow cytometry experiments to determine whether *aft1Δ* mutant cells show defects in cell cycle progression compared to wild type cells. Surprisingly, the results suggest that *aft1Δ* mutants do not show dramatic defects in cell cycle progression compared to a wild type control (Fig. 10, top two panels). These findings suggest that either Aft1p is not required for cell cycle progression or alternatively, the effects of Aft1p are minor, and undetectable by these methods.

To increase the sensitivity of the assay, I repeated the alpha factor block and release flow cytometry experiments upon treatment with the DNA damaging agent methyl methanesulfonate (MMS). Several studies have shown that cells are most sensitive to MMS during progression through S phase (*63-65*), making this drug a valid choice to investigate progression through the initial stages of the cell cycle. Interestingly, even after MMS treatment, *aft1Δ* mutants do not show significant defects in cell cycle progression compared to a wild type control (Fig. 10, bottom two panels). This suggests that either the flow

Figure 10. *aft1Δ* mutants do not display cell cycle defects compared to a wild type control upon treatment with MMS. Alpha mating factor was added to log phase *MAT*a wild type (YKB1032) and *aft1Δ::kanMX6* (YKB673) cells grown in YPD at 25°C. After a 2.5-hour incubation (to arrest cells in G1 phase), cells were washed and resuspended in fresh YPD media. Cultures were split in half, and released from cell cycle arrest by resuspension in YPD or YPD + 0.035% MMS. Both aliquots were incubated, with shaking at 25°C, and samples were taken every 15 minutes for 4 hours. DNA was stained with propidium iodide and DNA content was assessed by flow cytometry. The flow cytometry profiles shown are representative of the indicated time points for one of three experiments, which displayed similar results. The flow cytometry profile for the initial cell population in log phase is in solid black and the experimental sample is a red line.


WT
aft1Δ
WT + MMS
aft1Δ + MMS
t=0 min
40
90
120
150
180
225

cytometry experiments are not sensitive enough to detect any minor changes in the cell cycle, or that Aft1p is not required for the repair of MMS-dependent DNA damage. Indeed, the former may be the case as *aft1Δ* mutant cells have not been identified in genome-wide, high-throughput MMS sensitivity screens (38, 66).

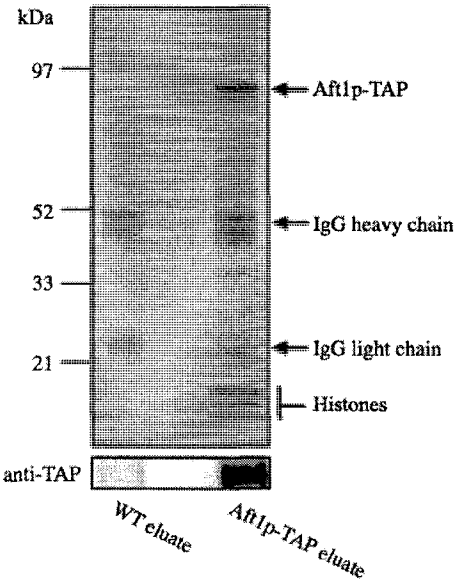
Affinity purification of Aft1p-TAP

To further explore the biological functions of Aft1p, a physical protein-protein interaction network was developed using affinity purification, followed by mass spectrometry analysis. Previous studies had not successfully isolated Aft1p by traditional Tandem Affinity Purification (TAP) methods (50, 51), likely because of the low abundance of Aft1p within the cell. One-step affinity purification, using IgG cross-linked magnetic beads and large quantities of whole cell extract (from 4 L of mid-log phase culture (OD_{600} ~ 0.6-0.8)), has allowed me to purify Aft1p-TAP expressed under its endogenous promoter to coomassie stainable levels (Fig. 11A). Western blot analysis with anti-TAP antibodies and identification of Aft1p by mass spectrometry confirms the purification of Aft1p-TAP. The purification procedure was performed in duplicate and Aft1p interacting proteins that were identified in both purifications by mass spectrometry, but were absent from the wild type control, are listed in Figure 11B.

Remarkably, the mass spec analysis revealed putative protein-protein interactions between Aft1p and five of the six subunits of the MCM complex (Cdc47p, Cdc54p, Mcm2p, Mcm3p, Mcm6p) which is known to play a role in DNA replication and is a presumed helicase at growing forks in yeast (*67*). Furthermore, the analysis identified a putative protein-protein interaction with Yku80p, a subunit of the Ku heterodimer which functions in

Figure 11. Identification of Aft1 protein-protein interactions.
(A) Affinity purification of Aft1p-TAP. Whole cell extracts of *MAT*a wild type (YKB1032) and Aft1p-TAP (YKB579) cultures were incubated on IgG coated magnetic beads overnight, with rotation at 4°C. Beads were isolated using a magnetic field and proteins were eluted off of the beads, run on a 4-12% pre-cast SDS-PAGE gel, and coomassie stained. Gel bands, encompassing the entire wild type and Aft1p-TAP tagged sample lanes were excised from the gel and proteins were identified by mass spectrometry. α-TAP Western blotting demonstrates the presence of Aft1p-TAP in the eluate. Gel is representative of one of two purification experiments, which displayed similar results. (B) Identified putative protein-protein interactions. All listed interactions appeared in two of two purifications and were absent from the wild type control.

A.



B.

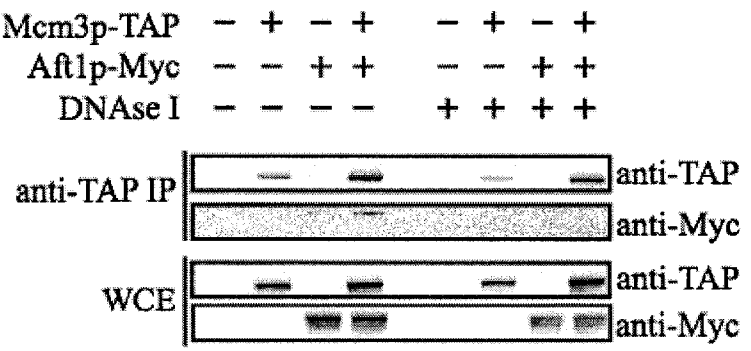
Protein	Function
MCM complex	DNA replication initiation
Cdc47p	
Cdc54p	
Mcm2p	
Mcm3p	
Mcm6p	
Transcription	
Rap1p	DNA binding protein
Rpo21p	RNA polymerase II largest subunit
Rpa49p	RNA polymerase I subunit
Rpa135p	RNA polymerase I subunit
DNA repair	
Yku80	subunit of Ku complex, dsDNA repair, telomere maintenance
Tra1	subunit of NuA4, DNA repair, histone acetylation
Other	
Pet9	major ADP/ATP carrier of mitochondrial inner membrane
Ssc1	subunit of SceI endonuclease, mitochondrial matrix ATPase
Cdc48	ATPase in endoplasmic reticulum

genome stability by participating in pathways for DNA double-strand break repair (*68*). These findings strongly correlate with the *AFT1* genetic interaction map which was enriched for genes involved in cell cycle regulation as well as DNA damage repair, thereby suggesting a functional connection between Aft1p and these two cellular processes.

The protein-protein interaction between Aft1p and Mcm3p is mediated through DNA

Having initially identified the physical interactions between Aft1p and five subunits of the MCM complex by mass spectrometry, I sought to test the validity of the interactions *in vivo* by reciprocal immunopurification of Aft1p using a TAP-tagged subunit of the MCM complex, Mcm3p. The enrichment for histones in the Aft1p-TAP purification, as well as the identification of putative physical interactions between Aft1p and the single-stranded DNA binding proteins Rap1p and Yku80p suggests that some of these interactions may be mediated through DNA. To test this, I generated a strain expressing both Mcm3p-TAP and Aft1p-Myc from their endogenous promoters and performed one-step affinity purification using IgG cross-linked magnetic beads with and without DNAse I treatment. As predicted, Aft1p-Myc did immunopurify with Mcm3p-TAP, but this co-purification was abolished upon treatment with DNAse I (Fig. 12).

Figure 12. The protein-protein interaction between Aft1p and Mcm3p is mediated through DNA. Whole cell extracts (WCE) were prepared from exponentially growing cells from the following *MAT*a strains: wild type (YKB1032), Mcm3p-TAP (YKB1014), Aft1p-Myc (YKB1016), and Mcm3p-TAP Aft1p-Myc (YKB1048). To determine whether interactions were dependent on DNA, WCE were treated with DNAse I as indicated. WCE expressing the indicated tagged proteins either untreated or treated with DNAse I (as indicated on top of panel) were immunoprecipitated with IgG coated magnetic beads. The immunoprecipitates (anti-TAP IP, top two panels) and WCE (bottom two panels) were then subjected to western blot analysis with anti-Myc or anti-TAP antibodies, as indicated on the right side of the panels. The figure is representative of one of three experiments, which displayed similar results.


Mcm3p-TAP − + − + − + − +
Aft1p-Myc − − + + − − + +
DNAse I − − − − + + + +
anti-TAP IP
anti-TAP
anti-Myc
WCE
anti-TAP
anti-Myc

Chapter 4: DISCUSSION

In this study I performed functional genomics and high-throughput proteomics to explore the biological roles of Aft1p under normal iron conditions. My data presented above provides evidence that Aft1p function is not limited to mediating the low iron transcriptional response. Rather, my work suggests that Aft1p contributes to numerous cellular processes under iron replete conditions including the Rim101 pathway, cell wall integrity, and DNA damage response. Although the mechanism by which Aft1p is involved in these cellular processes has yet to be elucidated, the results reveal novel insight into the multiple cellular roles that require Aft1p.

Genome-wide genetic screens identify diverse cellular roles for Aft1p

In an effort to further define the cellular functions of Aft1p, SGA methodology was used to perform complementary genome-wide SL and SDL screens (Fig.7 and Tables 2 and 3). As expected the genetic interaction map identified genes encoding proteins implicated in processes previously linked to Aft1p including iron regulation (*1*) and chromosome stability (*7*). The identification of these genes indicates that the *AFT1* SGA screens produced a high quality dataset. Interestingly, the genes identified in the *AFT1* genetic interaction map were not limited to the established roles for Aft1p, but predict possible functional roles for Aft1p in the Rim101 pathway, cell cycle progression, and DNA damage repair.

The wide range of cellular processes identified in the *AFT1* genetic interaction study suggests that either many of these processes are sensitive to cellular iron levels, or that Aft1p has additional iron-independent roles. To explore this possibility I asked whether *AFT2*, a paralog of *AFT1*, or *FET3*, a member of the iron regulon, shared genetic interactions with any of the genes identified in the *AFT1* SL-SGA screen. If *AFT2* and *FET3* shared a large

subset of genetic interactions with *AFT1*, I would hypothesize that the deletion mutants identified in the *AFT1* screen are likely sensitive to changes in cellular iron levels. However, *AFT1* and *AFT2* only shared three genetic interactions with the genes *SPE2*, a gene that encodes a protein required for the biosynthesis of spermidine and spermine (*61*), *MED1*, a gene that encodes a subunit of the RNA polymerase II mediator complex (*69*), and *YEL007W*, a gene that encodes a protein of unknown function (Table 6). In contrast, *FET3* identified eight shared genetic interactions with genes that encode for proteins involved in iron regulation, *FET4* and *CCS1* (*1*), genes that encode for members of the Rim101 pathway, *STP22*, *RIM20*, *RIM21*, and *RIM101* (*31*), as well as *SPE2* and *YEL007W*. Further, while four out of eight *FET3* genetic interactions displayed synthetic lethal phenotypes, all three *AFT2* genetic interactions displayed slow-growth phenotypes. These findings support the hypothesis that Aft2p has a weaker role in the transcriptional regulation of the iron regulon compared to Aft1p (*4, 26*). However, it is possible that Aft2p may have alternative cellular roles that are unique from its shared role with Aft1p in iron regulation. This hypothesis could be explored by performing genome-wide *AFT2* SL- and SDL-SGA screens. Lastly, the shared genetic interactions between *FET3* and *AFT1* with genes that encode for members of the Rim101 pathway suggest that cellular iron levels likely mediate the functional role of *AFT1* in this cellular pathway. In addition, these results suggest that the remaining predicted functions for *AFT1* identified by these screening methods are likely iron-independent.

Previous studies have demonstrated that SL and SDL screens are complementary in nature, with each screen identifying largely unique sets of genes (*7, 43*). As a result, genes identified in both screening techniques have been shown to be especially interesting and biologically relevant (*43*). As expected, the *AFT1* SL and SDL screens identified dramatically different data sets with only one genetic interaction with *SPE2* shared between

the two screens (Fig. 7). Interestingly, *SPE2* was also identified in the *FET3* and *AFT2* mini-SL screens suggesting that cells defective in spermidine and/or spermine biosynthesis may be hypersensitive to changes in cellular iron levels. The two amines, spermidine and spermine, are essential for aerobic growth in yeast, with *spe2Δ* mutants displaying similar phenotypes to cell division cycle (*cdc*) and actin mutants; including increased cell size, decreased frequency of budding, and delocalized chitin distribution (*61*). Aerobic respiration occurs in the mitochondria, with iron as an essential cofactor present in the heme of cytochromes and in iron-sulfur cluster proteins (*70*). This suggests that aerobic respiration is dependent on iron regulation within the cell, providing a potential link between the cellular role of Aft1p in iron regulation and the essential requirement for Spe2p for aerobic growth in yeast.

Aft1p and the Rim101 pathway

The most remarkable feature of the *AFT1* genetic interaction map is that several genes involved in the Rim101 pathway were isolated (Fig. 7). The Rim101 pathway is known to be involved in three cellular processes: alkaline pH response (*31*), sporulation (*28*), and cell wall assembly(*32*). Previously published results from the literature demonstrate that Aft1p is sensitive to alkaline conditions (*30*) and that Aft1p is required for proper sporulation (*62*), however a role for Aft1p in cell wall assembly had not been previously identified. I have determined that Aft1p is required for cellular resistance to SDS, caffeine, and calcofluor, three compounds that are known to cause growth defects in cell wall assembly mutants (*32*)(Fig. 8). These results demonstrate that Aft1p plays a functional role in all three cellular processes governed by the Rim101 pathway and suggest that these two proteins are functionally related.

In an effort to determine if Rim101p and Aft1p possibly share a role in additional cellular processes a genome-wide *RIM101* SL screen was performed (Fig.9). *AFT1* and *RIM101* only had five genetic interactions in common: *IMG2*, which encodes a mitochondrial ribosomal protein involved in the integrity of the mitochondrial genome (*71*), *MED1*, which encodes a subunit of the RNA polymerase II mediator complex (*69*), *SAC7*, which encodes a GTPase activating protein involved in signaling to the actin cytoskeleton (*72*), *MRE11*, which encodes a protein involved in the repair of DNA double strand breaks and telomere stability (*73*), and *YOR331C*, a potentially dubious open reading frame (ORF) of unknown function. Despite the limited overlap in *AFT1* and *RIM101* genetic interactions, both screens identified genes implicated in sporulation and cell wall assembly, cellular processes regulated by the Rim101 pathway. Surprisingly, *rim101Δ* mutants also displayed genetic interactions with three genes involved in DNA damage repair, including the previously mentioned *IMG2* and *MRE11*, as well as *MMS22*, which encodes a protein involved in preventing DNA damage caused by blocked replication forks (*74*). Furthermore, *rim101Δ* mutants are hypersensitive to the DNA damaging agents MMS (*75*) and cisplatin (*76*). This suggests that like Aft1p, Rim101p also participates in DNA damage repair by a yet uncharacterized mechanism.

The identification of a subset of genes involved in the Rim101 pathway in the *FET3* SL-SGA mini-screen suggests that the connection between Aft1p and Rim101p is iron-dependent. Yeast cells grow best in an acidic environment, as a proton gradient is crucial for the uptake of many nutrients (*77*). Upon alkaline conditions, yeast must adapt its gene expression profile to survive (*78*) including induced expression of genes involved in the acquisition and metabolism of copper and iron cations (*27-29*). This increase in expression is required because both copper and iron show a reduced solubility at alkaline pH (*30*).

Therefore, it is evident that an increase in pH results in the activation of the Rim101 pathway as well as increased expression of the iron regulon. However, it is unclear as to whether these cellular responses are connected in a linear fashion, with an increase in pH activating the Rim101 pathway which in turn results in increased expression of the iron regulon through the activation of Aft1p (or vice versa), or whether an increase in pH results in the activation of both of these cellular responses together. To investigate this, Northern blot analysis could be performed looking at the expression of genes of the iron regulon upon normal versus alkaline growth conditions in wild type, *aft1Δ*, *rim101Δ*, and *aft1Δrim101Δ* mutant strains. This would demonstrate whether the increased expression of the iron regulon is dependent on the presence of Rim101p.

An unidentified role for Aft1p in cell cycle regulation

An Aft1p physical interaction network was developed to provide further insight into the molecular mechanisms mediating the diverse cellular functions of Aft1p. The most remarkable feature of the protein interaction network is that Aft1p shares putative protein-protein interactions with five of the six subunits of the MCM complex (Cdc47p, Cdc54p, Mcm2p, Mcm3p, Mcm6p) (Fig. 11). The MCM complex acts as a helicase, unwinding DNA during the initiation and elongation steps of DNA replication (*79*). This interaction is intriguing as *aft1Δ* mutants have cell size defects suggesting a delay in cell cycle progression (*8*) and *aft1Δ* mutants display synthetic genetic interactions with genes involved in cell cycle regulation (Fig. 7). Although these combined results suggest that Aft1p may have a role during replication, defects in cell cycle progression were not detected in *aft1Δ* mutant cells by flow cytometry (Fig.10). Furthermore, Aft1p and Mcm3p do not physically interact, but

rather the observed interaction is mediated through DNA (Fig. 12), suggesting that Aft1p and the MCM complex may bind in close proximity on DNA. Alternatively, the interaction may be non-specific, dependent on contaminating DNA in the purification procedure that binds to both Aft1p and the MCM complex.

Although my purification procedure is informative and can provide insights into the cellular function of a protein of interest, there are several limitations to this analysis. The presence of DNA in the affinity purification demonstrates that future experiments are required to determine which of the putative protein-protein interactions are mediated through DNA and whether these interactions are specific. Large-scale Aft1p-TAP purifications using DNAse I treated extracts may be fruitful in identifying proteins that directly interact with Aft1p. Another limitation of this technique is that previously identified known interactions between Aft1p and other proteins were not identified. However, as these interactors were identified via yeast two-hybrid and/or co-immunoprecipitation assays, assays that can identify weak or transient protein interactions, these interactors might not be identifiable by our methods.

An unidentified role for Aft1p in DNA damage repair

The identification of DNA damage response genes in the *AFT1* genetic interaction map (Fig. 7) as well as the putative protein-protein interaction between Aft1p and Yku80p (Fig. 11), a subunit of the Ku heterodimer which functions to maintain genome stability through participation in DNA double-strand break repair (*68*), suggests that Aft1p may participate in DNA damage repair by a yet uncharacterized mechanism. However, defects in cell cycle progression upon treatment with the DNA damaging agent MMS were not

observed in *aft1Δ* mutant cells by flow cytometry (Fig. 10). These results suggest that either Aft1p does not have a role or only a minor role in MMS DNA damage repair that is undetectable by flow cytometry. Alternatively, Aft1p may be important for DNA repair caused by other DNA damaging agents. Indeed, a recent genome-wide drug sensitivity screen found that *aft1Δ* mutant cells are hypersensitive to inter-strand cross-linking DNA damaging agents such as carboplatin (*38*). It is also possible that the role for Aft1p in DNA damage repair is transcriptional. Microarray studies using *aft1-1* cells, a mutation that makes Aft1p constitutively localized to the nucleus, have identified a large number of genes that are transcriptionally regulated by Aft1p, most of which are not members of the iron regulon. Some of the identified genes have roles in DNA damage and checkpoint repair (*MAD*s and *BUB*s) (*6*).

Although these results suggest that Aft1p has a biological role in DNA damage response, it is unclear as to whether this cellular role is iron-dependent or independent. Hypersensitivity of *aft1Δ* mutants to the ribonucleotide reductase inhibitor HU can be suppressed by increasing levels of iron in the growth medium (*36*). In addition, there are DNA damage response proteins in yeast whose function is regulated by iron, such as the nucleotide excision repair helicase, Rad3p (*40*). In contrast, a genome-wide screen assessing the sensitivity of nonessential gene mutants to 12 different DNA damaging agents identified a hypersensitivity of *aft1Δ* mutant cells to inter-strand cross-linking DNA damaging agents, but did not isolate other members of the iron regulon (*38*). Furthermore, my *AFT2* and *FET3* SL-SGA mini-screens did not identify genes encoding proteins involved in DNA damage repair (Table 6). These conflicting results demonstrate that Aft1p may have iron-dependent and iron-independent roles in DNA damage repair however further analysis is required.

Conclusions

The objective of this study was to systematically identify the diverse cellular processes requiring Aft1p using an integrative genomics and proteomics analysis. The results confirm a role for Aft1p in iron regulation as well as chromosome stability, and provide further insight into the additional roles requiring Aft1p within the cell, including alkaline pH response via the Rim101 pathway, cell wall assembly, cell cycle regulation, and DNA damage repair. Although these experiments were performed under normal iron conditions, it is likely that the cellular role for Aft1p in the *RIM101* pathway as well as response to DNA damage is in some way dependent on cellular iron levels. Genes identified in the genetic interaction network and proteins identified in the physical protein interaction network represent a starting point for elucidating the cellular mechanisms behind Aft1p function in all of these cellular processes.

Future Directions

This systems biology analysis can be expanded through a series of secondary genomic screens and proteomics methods to predict which of the identified Aft1p biological functions are dependent on cellular iron levels. If *AFT1* genetic interactions are iron-dependent, I would expect mutants of other members of the iron regulon to display similar genetic interactions to those of *AFT1*. To test this hypothesis, genome-wide SL- and SDL-SGA screens can be performed with nonessential genes representing each subgroup of the iron regulon – *ARN1* for the siderophore transport system, *FET3* for the elemental iron transport system, *SMF3* for the vacuole iron recovery system, and *ISU1* for the iron-sulfur biosynthesis system (*1*). If there is no significant overlap in the genetic interaction profiles,

this would suggest that like Aft1p, other members of the iron regulon are multifunctional. If this is the case, any identified multifunctional proteins could be further explored using a similar integrative analysis as that employed in this study.

The proteomics methods can also be adapted to predict which of the identified Aft1p biological functions are dependent on cellular iron levels. If Aft1p has cellular roles under both iron-replete and iron-deplete conditions, I would expect that Aft1p protein-protein interactions will change upon different cellular conditions. To test this hypothesis, large-scale Aft1p-TAP purification using extracts grown under low iron versus high iron conditions may be fruitful in identifying which protein interactions are iron-dependent. Furthermore, affinity purification experiments can be performed using nuclear extracts instead of whole cell extracts to determine which proteins interact with Aft1p when it is present in the nucleus. This extended physical interaction network may provide substantial insight into the molecular mechanism by which Aft1p mediates its multiple cellular roles.

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Appendix A. Reagents, solutions, and buffers

Chemicals and Reagents:

- Benomyl (Sigma, 38, 158-6)
- BPS (Sigma, B1375-1G)
- $FeSO_4$ (Sigma, F8048-500G)
- ClonNAT (Werner BioAgents, 5.0000)
- Kanamycin (Sigma, A1720)
- Canavanine (Sigma, C1625)
- Caffeine (Sigma, C0750)
- Calcofluor (Sigma, F3543)
- α-factor (Sigma, T6901)
- Methyl methanesulfonate (Sigma, M4016)
- Propidium iodide (Sigma, P-4170)
- Protease inhibitor cocktail (Sigma, P8215)

Enzymes:

- RNAse A (Sigma, R-5125)
- Proteinase K (Invitrogen, 25530015)
- Lyticase (Sigma, L2524-200 KU)
- DNAse I (Sigma, DN25)

Antibodies:

- Rabbit IgG (Chemicon, PP64)
- α-TAP (Open Biosystems, CAB1001)
- α-Myc (Roche, 11667149)
- Peroxidase-conjugated goat α-mouse IgG (BioRad, 170-6516)
- Peroxidase-conjugated goat α-rabbit IgG (Chemicon, AP307P)

Affinity Purification:

- Lysis buffer:
  20 mM HEPES pH 7.4
  0.1% Tween-20
  2 mM $MgCl_2$
  300 mM NaCl
- Glass beads (Fisher Scientific, 35-535)
- Magnetic dynabeads (Dynal, Invitrogen 143-01)
- 1 x loading buffer:
  50 mM Tris pH 6.8
  2% SDS
  0.1% bromophenol blue
  10% glycerol
  2% 2-mercaptoethanol
- 4-12% pre-cast polyacrylamide gradient gel (Invitrogen, NP0321BOX)
- 1 x MOPS buffer

- Coomassie stain:
  Novex Colloidal Blue Stain Kit (Invitrogen, 46-7015, 46-7016)

Immunoblotting:

- Bjerrum and Schafer-Nielsen transfer buffer (1986):
  48 mM Tris base
  39 mM glycine
  20% methanol
  0.0375% SDS
- 0.1% PBS-T:
  1 mL of Tween-20 in 1 L of 1 x PBS
- 5% blocking solution:
  1 g skim milk powder
  up to 20 mL with 0.1% PBS-T
- ECL Plus Western Blotting Detection System (Amersham BioSciences, RPN2135)

Appendix B. Yeast strains used in this study

Strain	Auxotrophies	Reference
YKB579	*MATa AFT1-TAP*	Invitrogen
YKB673	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1, aft1Δ::KanMX*	(*7*)
YKB676	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, aft1Δ::NAT*	This study
YKB731	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his 3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+*	Gift from C. Boone
YKB779	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	(*80*)
YKB794	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, pGAL1-AFT1*	This study
YKB795	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, pRS416*	This study
YKB1008	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, rim101Δ::NAT*	This study
YKB1009	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, fet3Δ::NAT*	This study
YKB1010	*MATα can1Δ::STE2pr-Sp-his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+, aft2Δ::NAT*	This study
YKB1014	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, MCM3-TAP::HIS*	TAP collection
YKB1016	*MATa AFT1-13Myc::KanMX*	(*7*)
YKB1048	*MATa MCM3-TAP::HIS AFT1-13Myc::KanMX*	This study

AWARDS (con't)

Western Scholarship of Excellence, University of Western Ontario **2001**
London, ON
- Awarded for academic excellence; admissions average 90-93.9%

Canadian Millennium Scholarship Foundation Award of Excellence **2001**
- Recognizes students' academic achievement, contributions to the community, leadership and interest in innovation

Lieutenant Governor's Community and Volunteer Award, **2001**
A.Y. Jackson S.S.
Kanata, ON
- Recognizes outstanding volunteer activity among the graduating students of each of Ontario's secondary schools